DOCTOR OF PHILOSOPHY

Evolutionary biology of the Western rattlesnake, (Serpentes: Viperidae: Crotalus viridis) : phylogeny, morphology, and venom evolution

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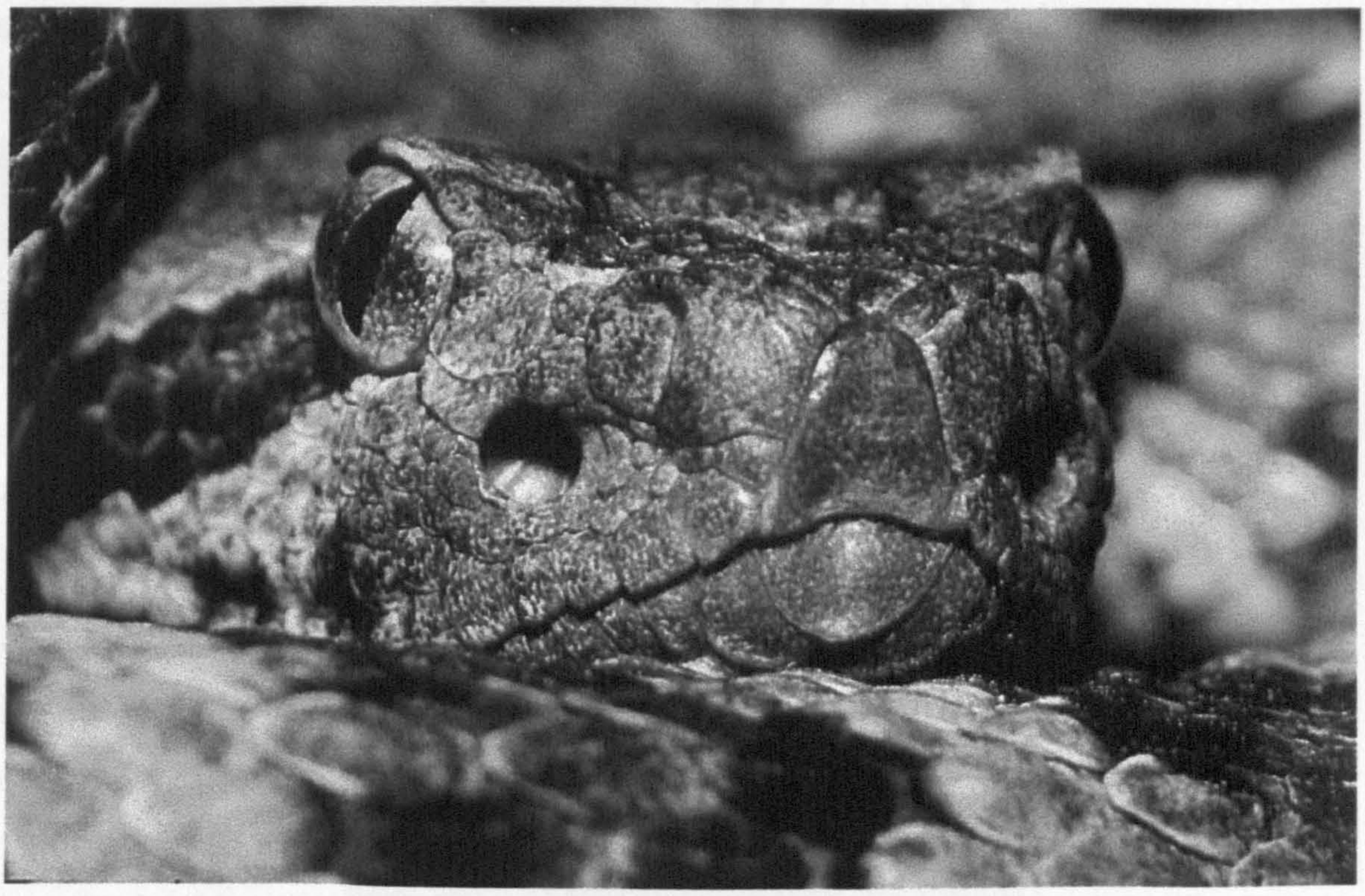

A thesis submitted to the University of Wales, Bangor by

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Summary

A multidisciplinary hypothesis testing approach is adopted to investigate the intraspecific relationships, and venom evolution within the polytypic species, *Crotalus viridis*, in western North America. A molecular phylogeny is reconstructed from mitochondrial cytochrome *b* (678 bp) and NADH dehydrogenase subunit 4 (ND4; 669 bp) DNA sequence information. The phylogeny is not concordant with the conventional subspecies categories, but shows strong geographical structuring corresponding to the major geographic regions of the western U.S. The basal split gives rise to two lineages (5.1-6.4% sequence divergence) corresponding to haplotypes east and west of the Rocky Mountains. Within the western lineage, *Crotalus viridis cerberus* forms a sister group to the other western haplotypes, and appears to have a long, independent evolutionary history.

Multivariate morphometric analysis also reveals regional structuring. Clear eastern and western forms are apparent, although these populations are not totally reproductively isolated. North-south clinal variation in morphology is found among populations east of the Rocky Mountains between Montana and Arizona. Among the western forms, there is clearly a zone of intergradation between the Great Basin and Pacific Coast forms, represented by a morphological cline in Idaho. Clinal variation was also found between the northern and southern Pacific Coast forms.

Venom evolution is of interest in *C. viridis*, since *C. v. concolor* is the only subspecies of *C. viridis* to secrete a high toxicity PLA_2 phospholipase (Concolor toxin) in its venom in adulthood. Isoelectric focusing of venom proteins revealed 14 variable bands, of which only one was unique (pI 8.52) to *C. v. concolor*. Principal coordinates analysis revealed three main venom types, corresponding to the Pacific Coast, *C. v. concolor*, and the remaining populations respectively. However, *C. v. concolor* tends to cluster with the latter group when the unique venom band is excluded from the analysis.

Phylogeny, rather than ecology, appears to be an important cause of geographic variation in both morphology and venom, as revealed by partial Mantel tests. Many characters are influenced by both phylogeny and ecology, however, probably because many causes of variation are intercorrelated. It is suggested that selection on venom composition probably varies according to the function of individual components.

The systematics of *Crotalus viridis* complex is reviewed according the criteria of the general lineage concept of species. Combined evidence from the molecular phylogeny and morphology (and to a lesser extent venom) suggests the existence of three species, to be named *Crotalus viridis*, *Crotalus cerberus*, and *Crotalus oreganus* (including the subspecies *C. o. oreganus*, *C. o. lutosus* and *C. o. concolor*).

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Title page photograph: *Crotalus viridis cerberus*, from nr. Payson, Gila County, Arizona. Same animal shown in figures 1.3 and 1.4, page 22. By W. Wüster.

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Abbreviations

3'	three prime
5'	five prime
A	Adenine
A	amps
ab	*Crotalus viridis abyssus*
AFLP	amplified fragment length polymorphism
ANCOVA	analysis of covariance
ANOVA	analysis of variance
bp	base pairs
C	Cytosine
ca	*C. v. caliginis*
ce	*C. v. cerberus*
CI	consistency index
cm	centimetre
co	*C. v. concolor*
CVA	canonical variate analysis
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dTTP	deoxythymidine triphosphate
ED_{50}	effective dose (mg antivenin/mg venom)
EDTA	ethylenediamine tetraacetate.$2H_2O$
ELISA	enzyme linked immunosorbant assay
ESU	Evolutionary Species Units
g	centrifugal force, equal to gravitational acceleration
g	gram
G	Guanine
he	*C. v. helleri*
HI	homoplasy index
HPLC	high pressure liquid chromatography
IEF	isoelectric focusing
i.p.	intraperitoneal
KH-test	Kishino-Hasegawa
km	kilometere
l	litre
LD_{50}	lethal dose of compound causing death in 50% of experimental animals
logL	log likelihood score
lu	*C. v. lutosus*
m	metre
M	molar
m.y.a.	million years ago
MALDI-TOF-MS	matrix assisted lazer desoprtion ionisation-time-of flight-mass spectrometry
ME	minimum evolution
mg	milligram (10^{-3} g)
ML	maximum likelihood
ml	millilitre (10^{-3} l)
mm	millimetre
mM	millimolar (10^{-3} M)
MP	maximum parsimony
mtDNA	mitochondrial DNA
mtDNA	mitochondrial DNA
mw	molecular weight
My^{-1}	per million years
µg	microgram (10^{-6} g)
µl	microlitre (10^{-6} g)
µM	micromolar (10^{-6} M)
n	number
NaAC	sodium acetate
ng	nanogram (10^{-9} g)
NJ	neighbor joining
nu	*C. v. nuntius*
°C	degrees centrigrade
or	*C. v. oreganus*
OTUs	operational taxonomic units
P	probability
PCA	principal component analysis
PCO	principal coordinate analysis
PCR	polymerase chain reaction
pH	acidity ($-\log_{10}$ [Molar conc. H^+ ions])
pI	isoelectric point
pmy	per million years
RC	rescaled consistency index
RE	restriction enzyme (endonuclease)
RFLP	random fragment length polymorphism
RI	retention index
RNA	ribonucleic acid
rpm	revolutions per minute
SDS PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SH-test	Shimodaira-Hasegawa test
spp	species
SPR	subtree pruning-regrafting
STE	saline-Tris EDTA
SVL	snout-vent (cloaca) -length
T	Thymine
TBE	Tris-borate EDTA
TBR	tree bisection-reconnection
TE	Tris EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
ti	transitions
Tris	tris (hydroxymethyl) amino ethane
tv	transversions
UPGMA	unweighted pair group method
V	volt (unit of tension)
vi	*C. v. viridis*
W	watt (unit of power)

Chapter 1. General introduction

"As touching Serpents, wee see it ordinarie that for the most part they are of the colour of the earth wherein they lie hidden: and an infinite number of sorts there be of them"- Pliny. Quoted by Laurence Klauber, in Rattlesnakes, Their Habits, Life Histories, and Influence on Mankind (1956), p183.

1.1 Systematics

The primary goal of systematics is the description of taxic diversity and phylogenetic reconstruction. The overall importance of this goal is that corroborated hypotheses of relationship are the prerequisites for all inferences regarding observed patterns of variation within the context of an historical perspective. This might involve patterns of geographic variation in a range of different characters, the distribution across lineages of certain behavioural, ecological, or morphological attributes, or the pattern of distribution of variation at the molecular, DNA level (Miyamoto and Cracraft, 1991).

1.1.1 *Geographic variation and the subspecies problem*

Geographic variation, the occurrence of differences among spatially segregated populations within species, is found in nearly all groups of organisms. The degree and patterns of geographic variation vary considerably for different species, and in some instances variation may be highly localised among neighbouring demes, for which the term microgeographic variation has been coined (Mayr, 1970; Gould and Johnston, 1972; Thorpe, 1987). The existence of geographic variation has been acknowledged since the Linnaean period or earlier (Mayr, 1970; Endler, 1977). Classical evolutionary and taxonomic thinking, however, was highly "typological" (Mayr,

1970). Taxonomists believed in arranging organisms into unvarying, discrete categories such as species, then subspecies. The acceptance of geographic variation to replace "typological thinking" was therefore pivotal in the development of the modern view of evolution (Futuyma, 1986). In turn many questions have been raised regarding species concepts (e.g. Wilson and Brown, 1953) and how the "species" unit should be defined, especially with the emergence of new perspectives arising from molecular analyses. The interpretation of patterns and potential causes of geographic variation has therefore become an integral part of contemporary intraspecific systematic studies.

Conventionally, subspecies have been used to describe geographic variation within single species (Thorpe, 1980a; Cracraft, 1989; Frost and Hillis, 1990; Shaffer and McKnight, 1996). Originating from the conceptual background of the biological species concept, subspecies represent morphologically differentiated but reproductively compatible populations within polytypic species. Subspecies are capable of interbreeding, and exhibit varying degrees of phenotypic variation from barely distinct to diagnosably distinct populations (Cracraft, 1989).

The recognition of the subspecies category is controversial (Wilson and Brown, 1953; Thorpe, 1987; Cracraft, 1989; Frost and Hillis, 1990; Shaffer and McKnight, 1996), and for several reasons. In most cases, descriptions do not follow strict, predefined criteria (Wilson and Brown, 1953; Cracraft, 1989; Frost and Hillis, 1990). Rather, subspecies categories often represent arbitrary subdivisions of geographic variation, or "slices of clines", without consideration of congruence between characters or the underlying causes of that variation (Thorpe, 1987; Cracraft, 1989; Frost and Hillis, 1990). In this respect subspecies cannot qualify as objective, evolutionary units (or the entities which speciate), as popular opinion previously upheld (Cracraft, 1989). Moreover, subspecies are usually initially defined on the basis of a single, superficially obvious difference, while incongruent characters are conveniently ignored (Thorpe, 1985a; Cracraft, 1989). For example reptile subspecies are often described on the basis of differences of colour pattern, size, and superficial differences in scale counts, as is the case in the western rattlesnake, *Crotalus viridis* (Klauber, 1956), the garter snake

Thamnophis sirtalis (Conant, 1975; Benton, 1980), and whiptail lizards, *Cnemidophorus tigris* (Taylor and Buschman, 1993). Benton (1980) points out that there would be a great and impractical proliferation of subspecies if this practice was extended to all species. The criterion of reproductive compatibility is of dubious use: shared reproductive compatibility is a symplesiomorphy, and therefore does not provide evidence for monophyly (Cracraft, 1989). Furthermore, reproductive compatibility among allopatric forms cannot be tested meaningfully, because pre-mating barriers can break down in unnatural conditions (Mayr, 1975; Cracraft, 1989). Inevitably, therefore, the splitting of populations into subspecies based on superficial differentiation often results in misleading phylogenetic interpretation. Subspecies generally offer little or no clue to the cause of the variation other than a false and circular assumption that physiographic features are the cause (Klauber, 1935, 1949; Thorpe, 1980a, 1987). The circularity arises from the arbitrary partitioning of a species according to some physiographic feature, thus giving the misleading impression to other workers, that the feature is the actual cause of differentiation within the species.

The major casualties of subspecies splitting tend to be widely distributed species exhibiting pronounced intraspecific geographic (phenotypic) variation, and species possessing any number of island populations (Wilson and Brown, 1953; Thorpe, 1980a; Wüster and Thorpe, 1992). In the past, polytypic species have presented a major systematic challenge, and in many cases the relationships of the different populations remain untested and unrevised for long periods. The consequence of this tends to be what Good (1994) termed the "inertial species concept", whereby for prolonged time periods, the populations of polytypic species are simply treated as conspecific out of habit, as opposed to positive evidence of conspecificity.

Numerous contemporary morphological and molecular studies of wide-ranging, polytypic reptile species have reported patterns of geographic variation in a range of characters that do not correspond to the conventional subspecies. For example in the garter snake, *Thamnophis sirtalis* (Benton, 1980), the ringed snake, *Natrix natrix* (Thorpe, 1984), *Anolis* lizards (Malhotra and Thorpe, 1991a, 2000a), the ridgenose

rattlesnake, *Crotalus willardi* (Barker, 1992), the rock rattlesnake, *Crotalus lepidus* (Dorcas, 1992), the *Vipera ursinii* complex (Joger *et al.*, 1992), Russell's viper, *Daboia russelii* (Wüster *et al.*, 1992a), and whiptail lizards, *Cnemidophorus tigris* (Taylor and Buschman, 1993). Instead the subspecies have been found to represent trivial local races, composite populations of several evolutionary lineages, or have warranted separation into several distinct species.

The need to adopt a robust hypothesis-testing procedure, with clear statements of the criteria used, is paramount in resolving species systematics (Sites and Crandall, 1997), since disregard for species status has serious implications with respect to species conservation and assessing biodiversity (e.g., Bernatchez and Wilson, 1998; McElroy, 1995; Avise *et al.*, 1998, Puorto *et al.*, 2001), and in the case of the venomous snake species, toxinological research and the development of efficient antivenoms for the treatment of bites (Wüster and Thorpe, 1991; Wüster and Thorpe, 1992; Wüster *et al.*, 1992a; Wüster and McCarthy, 1996).

1.1.2 *Species concepts*

A crucial element in any systematic study is the definition of a species. This is not straightforward, however, and over many years, biologists have established a minor industry devoted to the production of alternative species concepts. Until recently the multiplicity of species concepts has only served to confound the formulation of any unified species definition (De Queiroz, 1998).

The various species definitions reflect the diverse types of evolutionary questions and/or organisms with which their authors were primarily concerned (Templeton, 1989). To complicate matters further, many species concepts suffer the same problems of synonymy as species themselves, in that a given concept may also be known by several different names. For several decades prior to the advent of molecular phylogenetics, the biological species concept (e.g., Mayr, 1942; Mayr, 1969) was

widely followed, which emphasised interbreeding among populations of a species, but reproductive isolation from other species. Derivatives of this concept include the isolation species concept (e.g. Mayr, 1963) which emphasises limited gene exchange due to reproductive isolation by natural mechanisms, and the recognition species concept (e.g., Paterson, 1985), which emphasises the common fertilisation and specific mate recognition systems shared by conspecific organisms.

Whereas the biological and recognition species concept focused on ongoing processes of genetic exchange, other, more recent species concepts focused on the nature of species as historical lineages. The evolutionary species concept was proposed to account for the observation that some populations appear to maintain distinctness despite interbreeding with other populations, and to acknowledge that asexual organisms also form species (e.g., Wiley, 1978). A related concept to the evolutionary concept is the phylogenetic species concept (e.g., Cracraft, 1983). This term arose from the ideology of phylogenetic systematics or cladistics, and has been applied to at least three different species definitions, including the "cladistic species concept" (Ridley, 1989), species concepts based on monophyly (de Queiroz and Donoghue, 1988; Donoghue *et al.*, 1992), and the diagnostic approach (Cracraft, 1983; Baum and Donoghue, 1995). Other concepts include the ecological species concept (Van Valen, 1976), the cohesion species concept (Templeton, 1989), the phenetic species concept (Sneath and Sokal, 1973). For a detailed review see De Queiroz (1998).

In an attempt to unite the diversity of concepts and theories, De Queiroz (1998) proposes that all species concepts are variations of a general, single theme of species as evolutionary lineages. He suggests that the complications of species definitions arise not from the multitude of concepts, but from the variations in the criteria underlying those concepts. Hence, different criteria provide information on different phenomena associated with the separation of lineages, and different researchers emphasise different criteria, based on their research priorities. Consequently, rather than relying on one criterion as the single pivotal factor, it would be more useful to

test the observed data against the predictions of all the various criteria used, to determine if they fulfil any or all of them.

De Queiroz's idea of the general lineage concept is both realistic and easily applied. Each of the various criteria for the diagnosis of species make requirements and predictions about patterns of variation to be observed in morphology, mtDNA or other genetic marks, or both, as summarised in Table 6.1, General Discussion (based on the summaries provided by de Queiroz, 1998 and Puorto *et al.*, 2001).

1.2 Investigative approaches to systematics of complex groups

Three contrasting approaches have been found useful in investigating the systematics of complex species groups, and the phenomenon of geographic variation. Multivariate morphometrics permit the analysis of patterns of variation in large numbers of characters simultaneously (Thorpe, 1976; Thorpe, 1980a); comparative mitochondrial DNA sequencing can reveal cryptic species and population phylogeny free of the confounding effects of natural selection on morphological traits (Thorpe, 1996); finally partial Mantel (matrix correspondence) tests (Manly, 1991; Mantel and Valand, 1970) can reveal significant associations between observed variation in the phenotype of the subject and hypothesised causes of this variation. Hypothesised causes may be historical, such as phylogeny (inferred from DNA sequence information) and vicariance biogeography (inferred from geology and vegetational history), or current natural selection for present-day ecological conditions (for example biotic and abiotic factors such as climate, habitat, diet) (Thorpe, 1975a; Endler, 1982; Fleischer and Johnston, 1982; Thorpe, 1987; Malhotra and Thorpe, 1991a; Wüster *et al.*, 1997).

1.2.1 Mitochondrial DNA and phylogeography

Molecular investigations of systematic problems have become a standard means of elucidating phylogenetic history (Hillis, 1987). The foremost benefit of molecular data is the extent of the data set. Nuclear genomes, coupled with their extranuclear counterparts, are exceedingly large and complex, offering the systematist an almost endless, diverse array of characters with different structural or functional properties, mutational/selectional biases, and evolutionary rates (Hillis, 1987; Miyamoto and Cracraft, 1991). Virtually any level of phylogenetic question can, therefore, be addressed depending upon which genome, and which portion within that genome, is chosen. Furthermore, comparable (orthologous) genes can be examined across virtually all living taxa, enabling the retrieval of phylogenetic record extending far back in evolutionary history (Hillis, 1987; Hillis and Huelsenbeck, 1992).

The increased interest in using DNA sequences in comparative work is attributable to the technological advances of the last few decades, including the polymerase chain reaction (PCR) (Saiki, 1988), the development of numerous "universal" primers (Kocher *et al.*, 1989), and DNA sequencing (Sanger, 1977). Automation of these procedures has enabled the very rapid generation of large molecular data sets (Kocher *et al.*, 1989; Miyamoto and Cracraft, 1991). The success of PCR in amplifying DNA from blood and fresh tissue, mummified, and possibly formalin-preserved tissues (e.g., Rosenbaum *et al.*, 1997; Su *et al.*, 1999; Chatigny, 2000), has also greatly enhanced the value of samples which were previously not accessible to the molecular systematist (Miyamoto and Cracraft, 1991).

Molecular data offer several advantages over morphological data. The fundamental difference is the contrast in the number of characters available for phylogenetic investigation. Many morphological characters are influenced by environmental factors, which may mask and confound the phylogenetic picture (Hillis, 1987; Thorpe *et al.*, 1991; Daltry *et al.*, 1996a). Second, it is very difficult to identify non-homoplasious

morphological characters, which is less of a problem with molecular characters (Hillis, 1987; Hillis *et al.*, 1996).

Mitochondrial DNA (mtDNA) is one of the most frequently used markers in animal molecular systematics (Avise, 1986; Moritz *et al.*, 1987; Hillis *et al.*, 1996; Sorenson *et al.*, 1999; Su *et al.*, 1999). The reason for this popularity lies in the unique properties of mtDNA which avoid some of the sources of ambiguity encountered with nuclear genes. The mtDNA molecule is small, with a high copy number per cell, and is easy to isolate and assay (Avise, 1986; Zevering *et al.*, 1991; Avise, 1994). The mitochondrial genome has a simple genetic structure, generally lacking complicating features such as introns, repetitive DNA and transposable elements (Avise, 1994). Occasional examples of duplications and pseudogenes have been reported however (e.g., Zevering *et al.*, 1991; Hoelzel *et al.*, 1994; Arctander, 1995; Kumazawa *et al.*, 1996), as well as other novelties (e.g., Macey and Verma, 1997; Macey *et al.*, 1999). Despite these occurrences, gene composition and order is considered to be relatively stable among closely related taxa (Zevering *et al.*, 1991; Wolstenholme, 1992; Kumazawa *et al.*, 1996).

Unlike nuclear DNA, mtDNA undergoes a straightforward mode of matrilineal transmission, without recombination or other genetic rearrangements. The mitochondrial genotype within an individual is, in nearly all cases, homoplasmic (all copies are identical). Cases of heteroplasmy (the coexistence of two or more mitochondrial genotypes within an individual) have been reported, although the majority of individuals will be effectively haploid as regards the number of types of mtDNA transmitted. Mitochondrial DNA evolves rapidly compared to nuclear protein coding genes, between 5 and 10 times faster than an average nuclear rate in primates (Brown *et al.*, 1979; Kocher *et al.*, 1989), although rate varies very greatly across sites (Avise, 1986; Hoelzel *et al.*, 1994; Rand, 1994; Yang *et al.*, 1994), and between taxa (Takezaki *et al.*, 1995; Zamudio and Greene, 1997). A commonly cited estimate of rate of base substitution in mtDNA is 2% My^{-1} (Brown *et al.*, 1979), leading to the highly controversial idea that mtDNA evolves in a clock-like fashion, and hence could

be used to estimate divergence times. However, rates of mtDNA evolution may also be influenced by body size and metabolic rate (Martin and Palumbi, 1993; Mindell and Thacker, 1996; Mindell *et al.*, 1996), so that mtDNA in ectotherms might evolve 5 times more slowly than in endotherms (Rand, 1994; Zamudio and Greene, 1997).

Irrespective of the molecular clock debate (for summary see Page and Holmes, 1998), the faster rate of evolution of mtDNA provides a significantly greater number of informative characters, essential for studies of lower taxonomic groups using sequence data, where sufficient phylogenetic resolution can rarely be achieved using the slower evolving nuclear sequences (Blouin *et al.*, 1998). At higher taxonomic levels, however, the problems of homoplasy and saturation in mtDNA sequence data become more apparent (Prychitko and Moore, 1997; Johnson and Clayton, 2000).

Haploidy and rapid evolution, together with the maternal, non-recombining, mode of mitochondrial DNA inheritance, confer additional advantages, particularly in intraspecific phylogenetic studies (Moore, 1995; Sorenson *et al.*, 1999; Avise, 1994). First, mitochondrial genes have smaller effective population sizes than nuclear genes, which renders them less prone to lineage sorting, and hence incongruence with the species tree (Moore, 1995; Johnson and Clayton, 2000). Second, many different matrilineal mtDNA haplotypes (alleles) exist, which can be ordered phylogenetically within a species, and which exhibit geographical structuring (Avise *et al.*, 1987; Avise, 1994). As a result, mtDNA sequence information has been used particularly intensively for phylogeographic studies, in which the distribution of mtDNA haplotype clades across the range of a species or species complex is used to infer the history of that distribution (Avise, 1994; Avise *et al.*, 1988; Riddle, 1996; Bernatchez and Wilson, 1998; Puorto *et al.*, 2001). Moreover, phylogeography has been used widely to infer species boundaries in species complexes (e.g., Sbordoni, 1993; Zamudio and Greene, 1997; Rodríguez-Robles and de Jesús-Escobar, 2000; Patton and Smith, 1994; Puorto *et al.*, 2001).

1.2.2 Multivariate morphometrics

Multivariate analysis is a widely used phenetic (numerical) approach, which attempts to describe generalised patterns of geographic variation and the actual, or implied, relative similarity of the OTUs (operational taxonomic units, or in numerical taxonomy, any item, individual or convenient group used for comparison or analysis - Lincoln *et al.*, 1984) through the simultaneous comparison of several characters (Thorpe, 1976; Thorpe, 1987). Typically, systematic hypotheses are multivariate in nature when considering morphometric data (Gould and Johnston, 1972; Willig *et al.*, 1986), hence a univariate approach used alone can lead to unreliable and erroneous conclusions concerning overall differences among groups (Willig *et al.*, 1986). A major advantage of the multivariate statistical approach is that observed patterns of variation can be described without any prejudgement as to the cause (Thorpe, 1987). Multivariate statistics have been usefully applied in numerous morphometric studies of a wide range of organisms, including fish (Saborido-Rey and Nedreaas, 2000; Murta, 2000), insects (Hymenoptera (Sheppard and Smith, 2000; Williams and Goodell, 2000); Orthoptera (Tatsuta and Akimoto, 2000), mammals (New Zealand fur seal - Bradshaw *et al.*, 2000), and amphibians and reptiles (salamanders - Birchfield and Bruce, 2000), snakes: (*Natrix* - Thorpe, 1975b; Thorpe, 1980b; Thorpe, 1987; *Daboia russelii* - Wüster and Thorpe, 1992; Wüster *et al.*, 1992b; *Naja* - Wüster and Thorpe, 1990; Wüster and Thorpe, 1992; Slowinski and Wüster, 2000) and lizards (*Anolis* - Malhotra and Thorpe, 1991a, 2000a; Losos, 2001; gekkonids - Bauer and Branch, 1995; *Cnemidophorus* - Taylor and Buschman, 1993; Taylor and Cooley, 1995; *Varanus* - Thompson and Withers, 1997; *Gallotia* - Thorpe, 1985b; Thorpe, 1985c; Hernández *et al.*, 2000). The recency of many of these studies also shows that the importance of morphometric investigation in systematics has not waned, despite the surge in popularity of molecular phylogenetics.

1.2.3 Matrix correspondence or correlation (Mantel) tests

The matrix correspondence or correlation (Mantel) test is a non-parametric method used to test for statistically significant associations (correlation) between two independent, *n* by *n* matrices of dissimilarity coefficients describing the same set of entities Mantel, 1967; Mantel and Valand, 1970; Sokal, 1979; Smouse *et al.*, 1986; Legendre and Fortin, 1989; Manly, 1991; Sokal and Rohlf, 1999). The procedure involves the construction of a null distribution by Monte Carlo randomisation, whereby the independent matrices are held rigid while the rows and corresponding columns of the dependent matrix are randomly permuted (Smouse, 1986).

While the Mantel test only allows a comparison among two variables, a partial Mantel test can be used to compare three or more variables in the form of distance matrices (Smouse *et al.*, 1986; Legendre and Fortin, 1989; Brown *et al.*, 1991). Useful descriptions and examples detailing the partial Mantel calculation are provided in Smouse (1986), Legendre (1989), and Manly (1991). The accuracy of the significance level of the observed values is dependent on the number of random permutations specified (Jackson and Somer, 1989; Manly, 1991; Malhotra and Thorpe, 1997a). Manly (1991) recommends one thousand randomisations for estimating a significance level of about 0.05, and 5,000 randomisations as a realistic minimum for estimating a significance level of 0.01. Other authors (Jackson and Somer, 1989) suggest a minimum of 10,000 for important biological studies. Given that the Mantel approach is frequently used when there is little *a priori* knowledge of the precise functional relations among variables, a certain amount of trial and error in the definition of variables may be required in most applications (Smouse *et al.*, 1986).

Partial Mantel tests have been usefully employed in numerous intraspecific studies for testing causal hypotheses of patterns of morphological geographic variation (refer to Chapters 3 and 4 for examples). The method allows the association between morphological characters (individual or compound) with various biotic or abiotic ecological hypotheses (for example climate, habitat, or biotope) (Thorpe, 1987;

Thorpe *et al.*, 1995) to be established with the phylogeny partialed out (where phylogeny is represented in the form of a patristic distance matrix) (Thorpe, 1987; Thorpe, 1996). Significant associations with ecological hypotheses imply ecogenetic adaptation by natural selection (ecogenesis), whilst a significant relationship with phylogeny suggests an historical underlying cause. Simultaneous Mantel tests can test for significance of association with individual causal hypotheses free of the effect of inter correlation between hypotheses (Thorpe, 1996). Mantel tests are used to test ecogenetic and phylogenetic causal hypotheses of geographic variation in morphology and venom of *Crotalus viridis* in Chapters 3 and 4 respectively.

1.2.4 Independent contrasts

A form of the comparative method modified by Felsenstein (1985a), independent contrasts, present an alternative approach to pairwise testing causal hypotheses of geographic variation, whilst incorporating a phylogenetic framework. The method relies on a bifurcating phylogeny with known branch lengths, and each character is assumed to be evolving by a Brownian motion model that is independent in each lineage. In terms of ecological genetics, the Brownian motion model refers to random genetic drift (Martins, 2000). The analytical process involves extracting a series of contrasts among species from the phylogeny, through the comparison of characters in adjacent branches with time (branch length). The contrasts are considered statistically independent and can be used in regression or correlation studies (Felsenstein, 1985a).

Independent contrasts have been used in intraspecific studies to examine the evolutionary relationships between venom and diet (Daltry *et al.*, 1996a), morphology and performance (Zani, 2000), and body size relationships in a host-parasite system (Morand *et al.*, 2000). Generally, independent contrasts are more important in interspecific studies, to which the method has traditionally been tailored (Thorpe, 1996). Currently, independent contrasts work by comparing continuous variables representing observed data and a causal hypothesis across a phylogenetic tree.

However, in intraspecific studies, it is necessary to take into account factors such as geographic distance, which can only be represented adequately in a matrix format. Consequently, a matrix comparison method is more appropriate for studies at the intraspecific level. Although principal components can be extracted from such matrices, this leads to considerable loss of information. (Thorpe, 1996). Independent contrasts cannot compare ecologically or morphologically distinct populations that are phylogenetically identical (Thorpe, 1996). Comments regarding the pros and cons of using comparative methods are given in Frumhoff and Reeve (1994), Martins (2000), and Losos (1999).

Additionally, independent contrasts are not deemed an appropriate analytical approach in this thesis, because causal investigation of morphological (Chapter 4) and venom variation (Chapter 5) uses several compound (generalised) characters best expressed as matrices, and in several instances requires comparison of more than two ecological hypotheses at a time. Furthermore, individuals rather than group means are used in the analyses, which requires that the effects of geographic proximity must be taken into account in all tests.

1.3 Geographic variation in venom composition

Geographic variation in snake venom composition is thoroughly documented (reviewed in Chippaux *et al.*, 1991), and manifests itself in different magnitudes across a number of levels, from high to low taxonomic levels (e.g., Jones, 1976; Wüster and Thorpe, 1991; Anderson *et al.*, 1993; Daltry *et al.*, 1996a, 1998), within populations (e.g., Adame *et al.*, 1990; Glenn and Straight, 1977, 1978) and individuals on a temporal and/or ontogenetic, seasonal or even sexual basis (e.g., Fiero *et al.*, 1972; Gubenšek *et al.*, 1974; Marsh and Glatson, 1974; Reid and Theakston, 1978; Chippaux *et al.*, 1982; Mebs and Kornalik, 1984; Mackessy, 1988).

The phenomenon of intraspecific variation in venom composition has become the focus of intensive research, due in part to the serious problems arising in relation to human health, but also from the implications affecting other areas of biochemical research in industry. The main differences in venoms between populations of a species tend to be explained by presence of a single or few components that either exist as a number of isomeric or homologous forms, or which represent exceptional components within one population of a species. For instance, a number of isomeric forms of the protein, myotoxin A, have been characterised from *Crotalus v. viridis* venom (Aird *et al.*, 1991; O'Keefe *et al.*, 1996; Nedelkov and Bieber, 1997). Other well known examples include a number of different phospholipase A_2 (PLA_2) homologues in the venoms of certain populations of vipers and rattlesnakes (Glenn and Straight, 1977, 1978; Rael *et al.*, 1992). These high lethality PLA_2 toxins cause serious myotoxic and neurotoxic pathology. Crotoxin was the first of the neurotoxic PLA_2s to be discovered in venoms from the South American rattlesnake, *Crotalus durissus terrificus*. However, levels of Crotoxin in venom from the Central American forms (*C. d. durissus*) appear to be age-related. In the adults, Crotoxin is secreted in such low concentrations as to become clinically irrelevant. In contrast, juvenile *C. d. durissus* venoms contain high concentrations of Crotoxin on a par with the South American forms, which may or may not be the same species. A related toxin, Mojave toxin, is secreted by the main population of the Mojave rattlesnake, *Crotalus scutulatus scutulatus* (Glenn and Straight, 1978), and another homologue, Concolor toxin, has been discovered in the adult venom of one subspecies of *Crotalus viridis*, *C. v. concolor* (Glenn and Straight, 1977). Further considerations regarding myotoxins and PLA_2s in *Crotalus* are addressed in Chapter 5. Variation may also exist in the relative concentrations of components supposedly common to all populations. Individual variation in electrophoretic behaviour, and phospholipase A and L-amino acid oxidase activity have been reported in the Indian saw-scaled viper, *Echis carinatus*, from a climatically, geographically, and nutritionally homogeneous habitat (Táborská, 1971). Geographic variation in biochemical properties of venoms from *Atropoides nummifer* (formerly *Bothrops nummifera*) have also been noted in Central America (Jiménez-Porras, 1964).

1.3.1 Variation in clinical manifestations of snake venom poisoning

Geographic variation in the physiological effects resulting from envenomation has also been reported. For instance, the venom of *Bitis gabonica* from Togo was found to show higher lethality in a rabbit model than venoms from other countries throughout the species range. Togo venom showed greater capacity to induce extrasystoles (disrupted heartbeat), otherwise, physiological effects of envenomation were generally consistent across locality, and did not seem to vary between the eastern and western subspecies (Hyslop and Marsh, 1991). A clinically relevant example is that of geographic variation in the symptomatology of Russell's viper (*Daboia russelii*) poisoning, across its range in Asia. Neurotoxic symptoms present in Sri Lankan bite victims (Jeyarajah, 1984; Phillips *et al.*, 1988), whereas pituitary haemorrhage (or Sheehan's syndrome) is characterstic of envenomation in Burma and India. There are reports of geographic variation within India (Jayanthi and Gowda, 1988) and additional symptoms of conjunctival oedema and increased capillary permeability in Burmese victims (Myint-Lwin *et al.*, 1985; Warrell, 1986). Neurotoxic proteins have been isolated from the Sri Lankan venoms and some (but not all) southern Indian venoms (Phillips *et al.*, 1988; Theakston, 1997; Prasad *et al.*, 1999). Indian venoms are up to ten times more necrotising than those from Thailand or Burma (Theakston and Reid, 1983). Geographic variation in *Daboia russelii* was first recognised by five subspecies, but these categories are now considered to be invalid. No correlation exists between morphology and symptomatology (Wüster *et al.*, 1992a).

1.3.2 Problems associated with intraspecific venom variation

The most important issue surrounding intraspecific venom variation concerns human health (Warrell, 1986; Wüster and Thorpe, 1991; Daltry *et al.*, 1996a; Munekiyo and Mackessy, 1998). Marked geographical differences in symptomatology confound reliable identification of the species responsible for a bite (Daltry *et al.*, 1997), leading

to inappropriate treatment (Williams and White, 1990). Moreover, the fact that the systematics of many species complexes is often not fully resolved, is of crucial importance with respect to development of effective antivenom treatments (e.g. *Daboia russelii* (Wüster *et al.*, 1992a) and *Crotalus viridis* (Glenn and Straight, 1977). The efficacy of antivenoms may vary according region (Jiménez-Porras, 1964; Warrell, 1986; Anderson *et al.*, 1993; Warrell, 1997). Even when the offending snake is correctly identified, a given antivenom may provide effective treatment in one region, but fail to neutralise venoms of the supposed same species from another region, due to differences in antigenic qualities of venoms between snake populations. For example, in the treatment of envenomation by *Daboia russelii*, Indian (Haffkine or Serum Institute of India) polyspecific antivenoms do not effectively neutralise the more neurotoxic Sri Lankan venoms (Theakston, 1997). Inadequate antivenom treatment, from a lack of specificity of antivenom to the individual venom of the snake, was also reported in a case of envenoming by *Echis* spp. Antivenom raised against Iranian *Echis* venom was used to treat large numbers of vicitims of *E. ocellatus* bites in north-eastern Nigeria, resulting in ineffective treatment and a significant increase in hospital case fatality (Warrell and Arnett, 1976). Snake venoms also represent an important natural source of chemicals for toxinological, biochemical, and biomedical research (Mebs, 1978; Russell, 1983; Daltry *et al.*, 1997). Intraspecific variation, therefore, may confuse experimental research in these areas. For example, geographic variation in venom composition has been found in *Calloselasma rhodostoma*, the Malayan pitviper (Daltry *et al.*, 1996a; 1996b), an important source of the enzyme ancrod (Arvin™), used in blood coagulation therapy. However, focusing on venom variation within a specific locality, or within individuals, would help to provide a better understanding of the biological function and evolution of venom components (Daltry, 1995).

A final consideration, the toxic effects observed in laboratory animal models may not reflect the actual effects inflicted either on humans (Hyslop and Marsh, 1991), or different natural prey types. The susceptibility of an organism to a particular venom is related to the level of sophistication of the circulatory system and immune system of

that organism, which varies between the major classes, such as birds, mammals, or reptiles (Kardong, 1996). Local adaptation for resistance to snake venoms have also been shown in prey species living in areas of high densities of rattlesnakes, as is the case with *Spermophilus beecheyi* (the California ground squirrel) living in close proximity to the northern Pacific rattlesnake, *Crotalus viridis oreganus* (Poran *et al.*, 1987).

Geographic variation in venom composition is clearly significant in a broad range of fields, including evolutionary ecology, systematics, and clinical pharmacology and treatment of snakebite. It would, therefore, be of considerable interest to test possible causal hypotheses in an attempt to elucidate the evolutionary processes governing venom variation. Venom characters may be influenced by the same ecogenetic and/or phylogenetic processes affecting other character systems, including morphology, as described in the preceding sections.

1.4 The western rattlesnake, *Crotalus viridis* Rafinesque

The Western Rattlesnake (*Crotalus viridis*) is an ideal subject for the study of geographic variation and its causes. This species occurs across a climatically and physiographically diverse range, from southern Canada to northern Mexico, and from Iowa to the Pacific coast, and is represented in all major vegetation zones (see Brown, 1997; MacMahon, 1997 and Whitney, 1996 for descriptions of vegetation types). *Crotalus viridis* exhibits notable geographic variation in morphology (Klauber, 1972; Macartney *et al.*, 1990), habitat preference, and behaviour (Klauber, 1972). The observed morphological variation in *Crotalus viridis* (adult colour pattern and size) has led to the description of nine subspecies (figures 1.1 to 1.10). Of particular interest is the presence of two dwarfed populations on the Colorado Plateau, currently known as *C. v. concolor* and *C. v. nuntius* (Klauber, 1972). In addition to the intrinsic interest of the patterns of variation in this species, it is also important to note that *Crotalus viridis* has become a popular study species in fields ranging from movement ecology

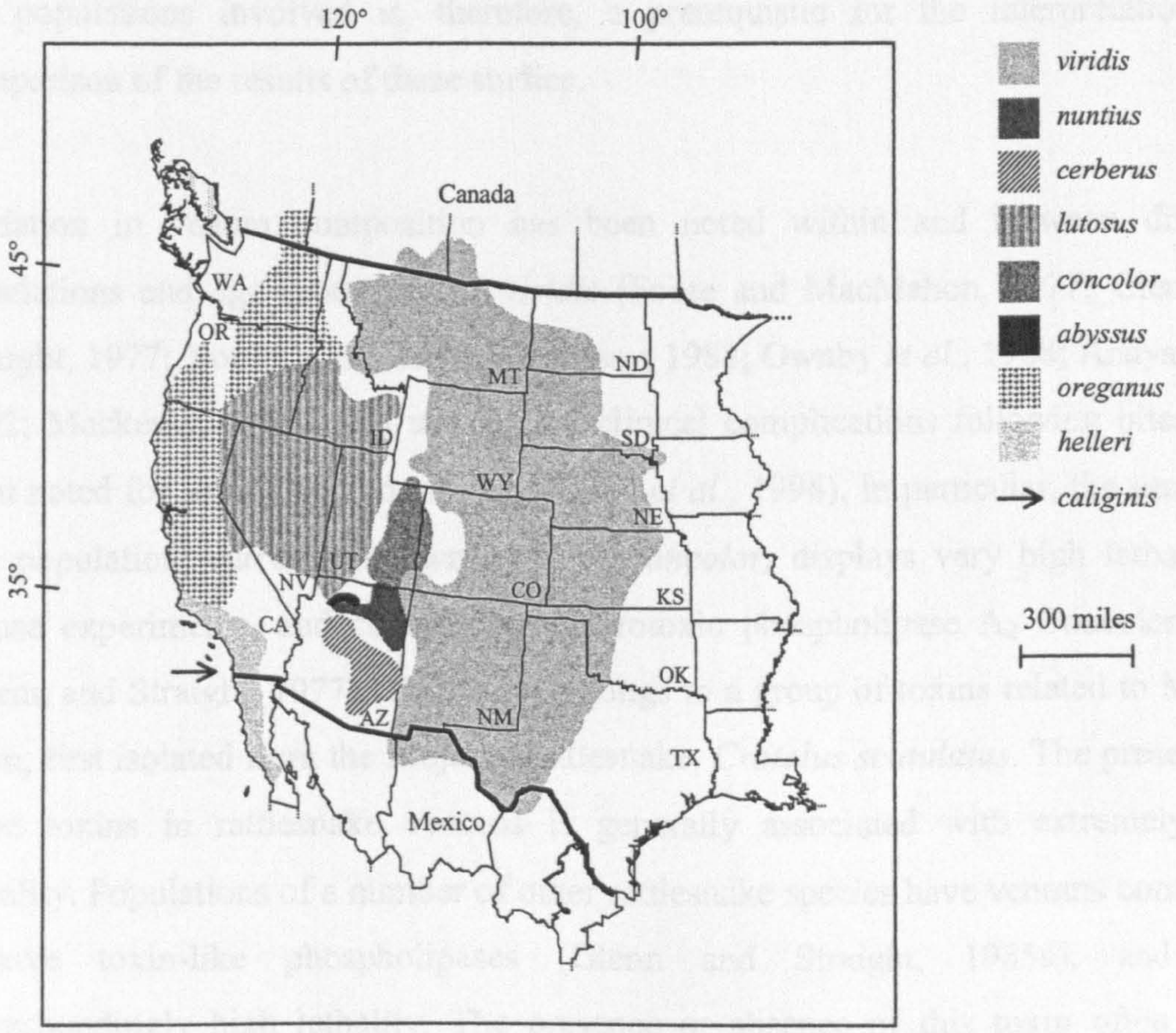


Figure 1.10. The distribution of the currently recognised subspecies of *Crotalus viridis* (after Klauber, 1972) in the western United states, northern Mexico and southern Canada. United States: WA=Washington, OR=Oregon, CA=California, ID=Idaho, NV=Nevada, AZ=Arizona, MT=Montana, UT=Utah, CO=Colorado, NM=New Mexico, WY=Wyoming, ND=North Dakota, SD=South Dakota, NE=Nebraska, KS=Kansas, OK=Oklahoma, TX=Texas.

(Schmidt, 1993; Duvall and Schuett, 1997) to feeding behaviours (Furry *et al.*, 1991; Kardong, 1993; Lavin-Murcio *et al.*, 1993; Hayes, 1995; Hayes *et al.*, 1995; Kardong, 1996; Duvall and Schuett, 1997), toxinology and pharmacology (e.g., Glenn and Straight, 1977; Young *et al.*, 1980; Aird and Kaiser, 1985; Aird *et al.*, 1988; Mackessy, 1988; Ownby *et al.*, 1988; Adame *et al.*, 1990; Aird *et al.*, 1991; Li *et al.*, 1993; O'Keefe *et al.*, 1996; Ownby *et al.*, 1997), and it has in many cases become a model organism representative of the Viperidae in general. Due to its wide range, different studies are often conducted in different parts of the range of the species, and

on different subspecies. A full understanding of the phylogeny and systematic status of the populations involved is, therefore, a prerequisite for the interpretation and comparison of the results of these studies.

Variation in venom composition has been noted within and between different populations and age groups of *C. viridis* (Foote and MacMahon, 1977; Glenn and Straight, 1977; Young *et al.*, 1980; Mackessy, 1988; Ownby *et al.*, 1988; Anaya *et al.*, 1992; Mackessy, 1993), and unexpected clinical complications following bites have been noted for some populations (e.g., Gibly *et al.*, 1998). In particular, the venom of one population, currently known as *C. v. concolor,* displays very high lethality in mouse experiments, and contains the neurotoxic phospholipase A_2 Concolor toxin (Glenn and Straight, 1977). This toxin belongs to a group of toxins related to Mojave toxin, first isolated from the Mojave Rattlesnake, *Crotalus scutulatus*. The presence of these toxins in rattlesnake venoms is generally associated with extremely high lethality. Populations of a number of other rattlesnake species have venoms containing Mojave toxin-like phospholipases (Glenn and Straight, 1985a), and with correspondingly high lethality. The presence or absence of this toxin often varies among different populations of one species. In *Crotalus viridis* populations other than *C. v. concolor*, these toxins are normally absent, except where possible hybridisation with *C. scutulatus* may have resulted in hybrid populations with the relevant genes (Glenn and Straight, 1990). Variation in venom components may be a function of phylogeny, natural selection for diet variation (as appears to be the case in *Calloselasma rhodostoma* - Daltry *et al.*, 1996a), or other factors. Despite the potential medical importance of the variability in the presence of Mojave toxin, there has so far been no intraspecific phylogenetic study of a rattlesnake species that includes both Mojave toxin-secreting and non-secreting populations.

1.5 Aim

The following study adopts a rigorous hypothesis-testing procedure with the purpose of reviewing the systematics and population phylogeny of *Crotalus viridis* using

molecular, multivariate morphometric, and venom data. In Chapter 2, the phylogenetic relationships of the *C. viridis* complex are investigated using mitochondrial cytochrome *b* and ND4 gene sequence data, and an attempt is made to elucidate the relationships of the taxa based on an historical interpretation of the results. Patterns of geographic variation in several morphological characters are examined by means of multivariate morphometrics, including principal component and canonical variate analyses (Chapter 3). Chapter 4 tests three ecological hypotheses, and one historical hypothesis (the mtDNA phylogeny from Chapter 2), proposed as possible causes giving rise to the observed patterns of variation in morphology in Chapter 3. Geographical variation in venom composition, pattern and cause, is explored in Chapter 5, with a view to providing a platform on which to base future venom research in *C. viridis*, including more rigorous causal hypothesis testing (e.g. diet), and more detailed comparisons of important venom components, particularly myotoxins. The systematics of the *Crotalus viridis* complex is reviewed in the General Discussion based on the evidence gathered from the preceding chapters. The taxonomic status of each conventional subspecies is revised in relation to the criteria of the general lineage concept of species, as outlined by de Queiroz (1998).

Figure 1.1. *Crotalus viridis nuntius*, from Angell, Coconino Co., Arizona. Photo: W. Wüster.

Figure 1.2 *Crotalus viridis viridis/nuntius*, from Old Highway 666, Apache Co., Arizona. This population could be regarded as intermediate between *C. v. viridis* and *C.v. nuntius*. Photo: W. Wüster.

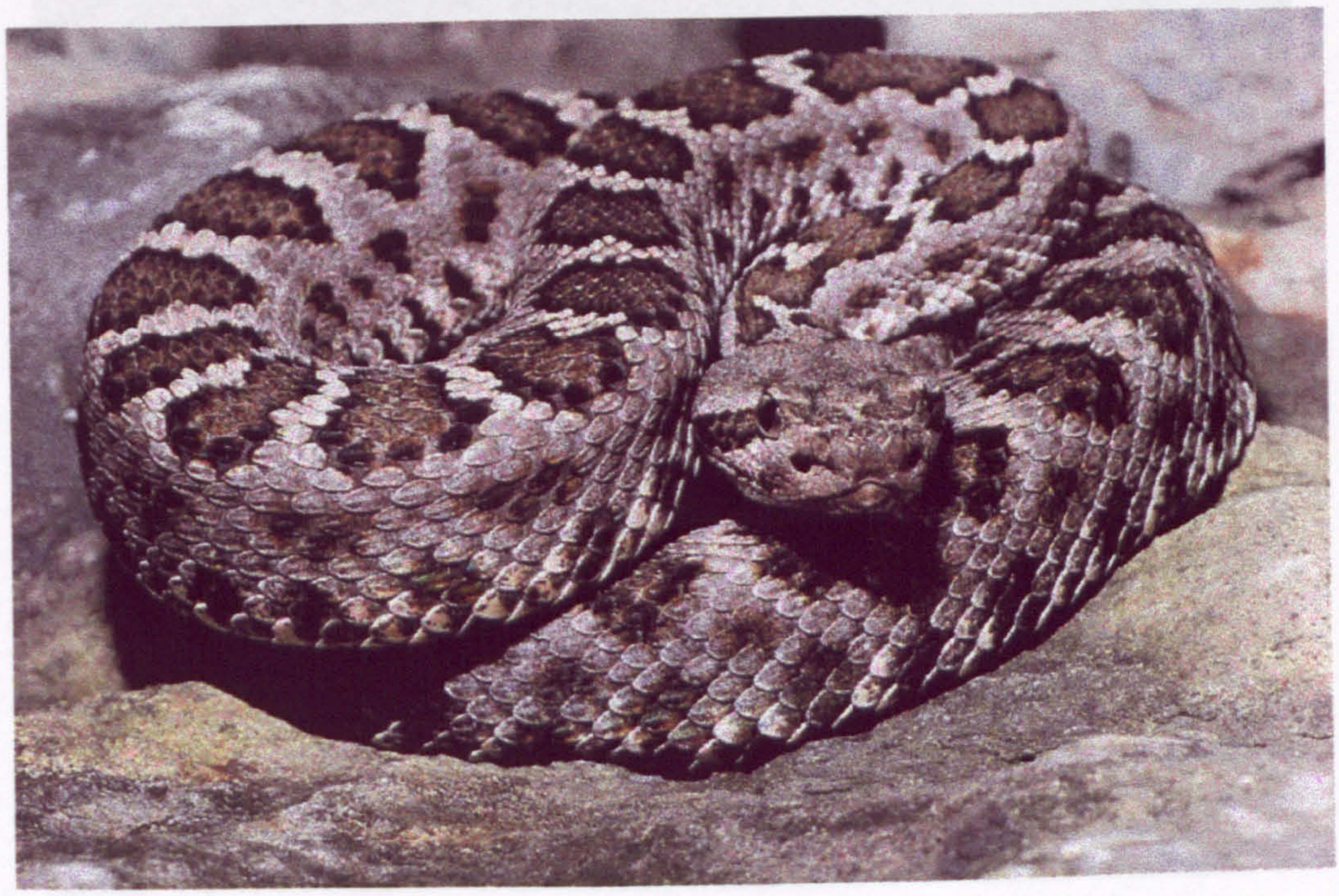


Figure 1.3. *Crotalus viridis cerberus*, half-grown specimen, creek off Highway 89, nr. Payson, Gila Co., Arizona. Note incipient melanisation of flanks. Photo: W. Wüster.

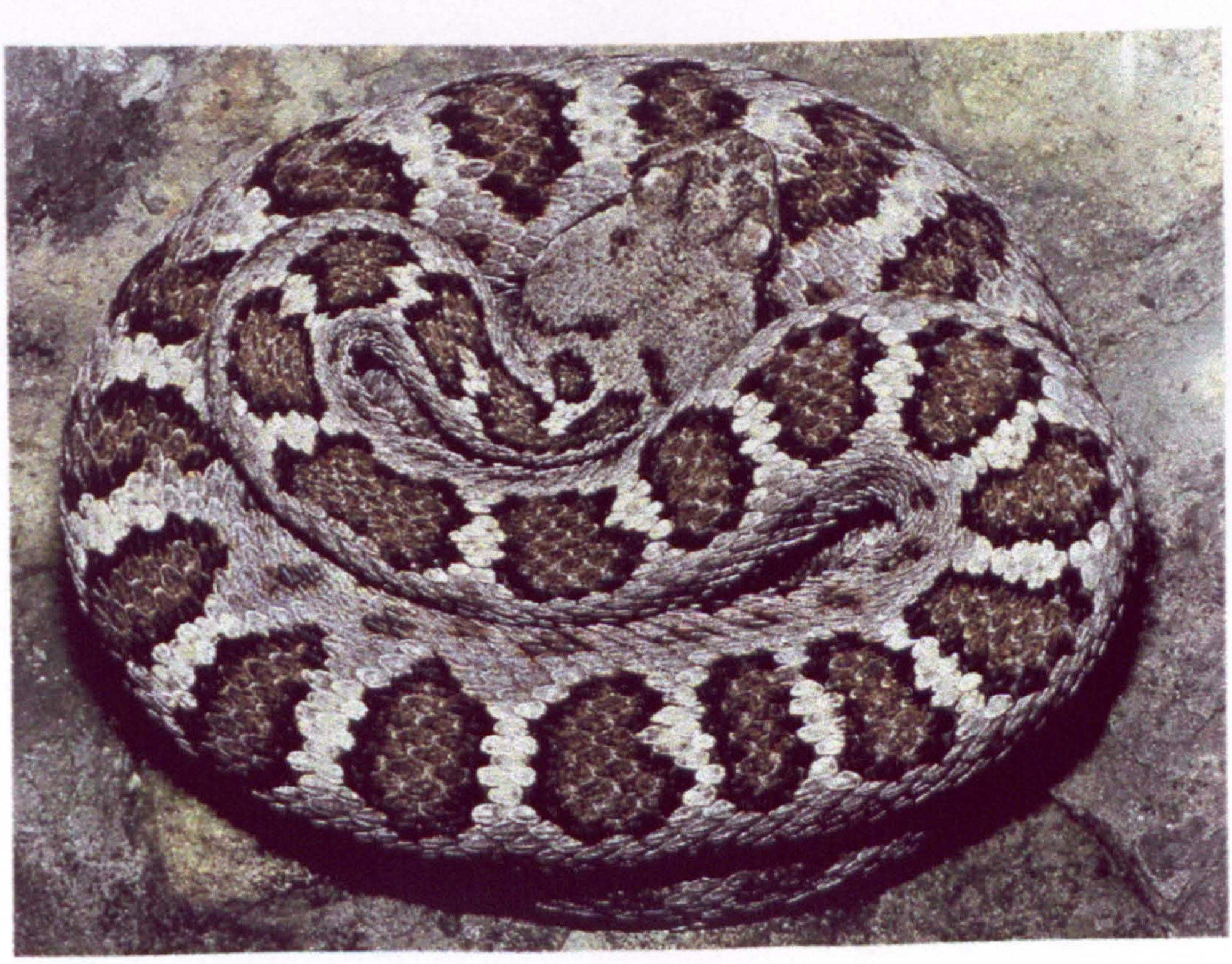


Figure 1.4. Same specimen as above. Note light bars between dorsal blotches, which remain visible even in many fully melanised, adult specimens. Photo: W. Wüster.

Figure 1.5. *Crotalus viridis oreganus*, brown specimen. San Francisco Bay Area, California. Photo: W. Wüster.

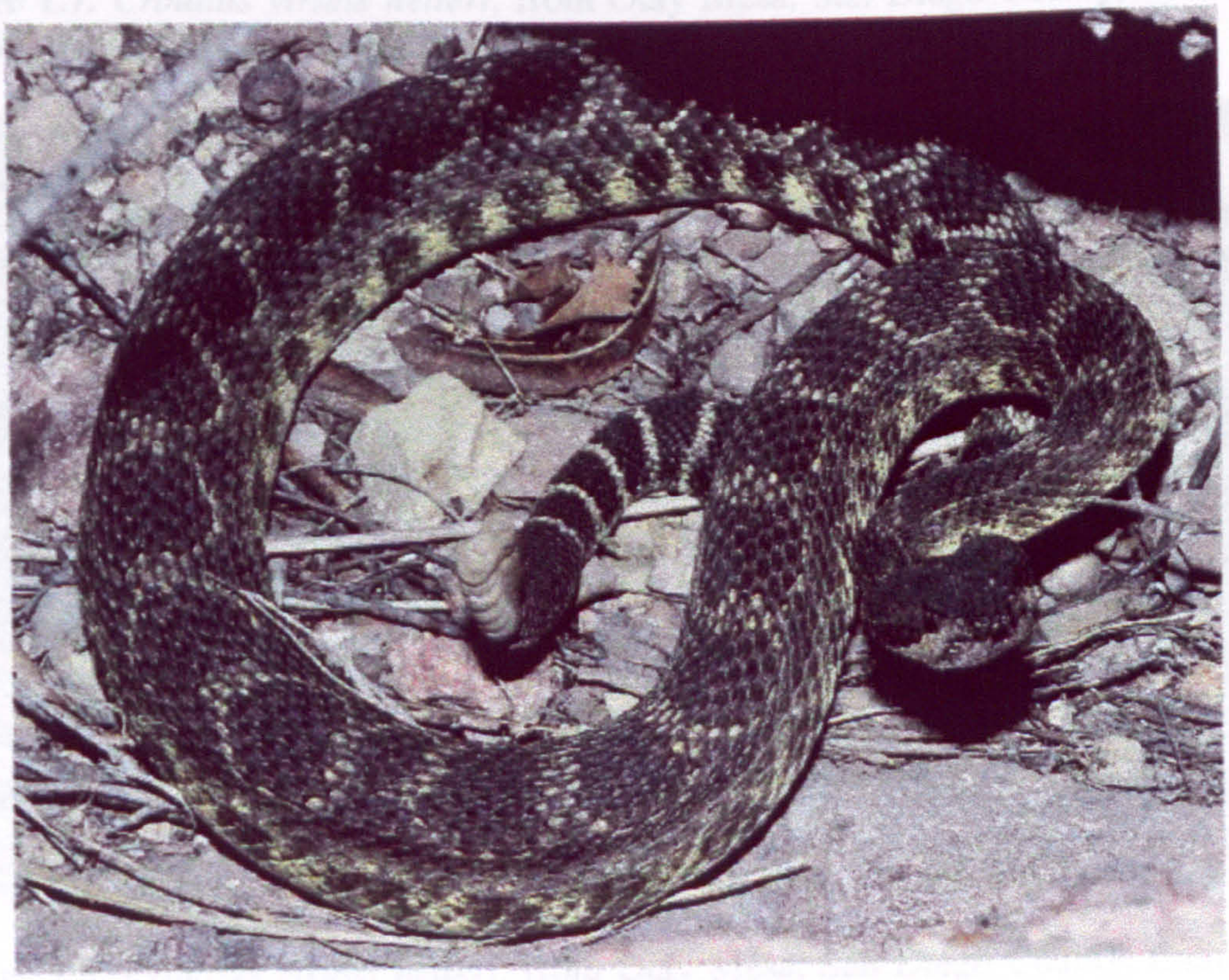


Figure 1.6. *Crotalus viridis oreganus*, dark specimen, Pinnacles National Monument, San Benito County, California. Photo: W. Wüster.

Figure 1.7. *Crotalus viridis helleri*, from Otay Mesa, San Diego County, California. Heavily melanised specimen. Photo: C. E. Pook.

Figure 1.8. *Crotalus viridis helleri*, from Otay Mesa, San Diego County, California. Compare contrast in colour pattern with melanised specimen in fig. 1.7 above. Photo: C. E. Pook.

Chapter 2. Phylogeny

2.1 Introduction

The properties of mtDNA and reasons for its popularity as a molecular marker have already been addressed in Chapter 1. Mitochondrial phylogenies have been used to examine the relationships of many taxonomic groups across the animal kingdom, including mammals - (Irwin *et al.*, 1991); reptiles - (Malhotra and Thorpe, 1994, 1997c, 2000c; Heise *et al.*, 1995; Herrmann and Joger, 1995; Pook and Wild, 1995; Reeder, 1995; Wüster *et al.*, 1999a; Pook *et al.*, 2000; Lenk *et al.*, 2001); birds - (Moum *et al.*, 1994; Freeland and Boag, 1999); fish - (Bernatchez and Wilson, 1998), and nematodes - (Blouin *et al.*, 1998). The widespread application of DNA information has also seen the development of sophisticated analytical packages, ultimately seeking to enhance retrieval of the optimum phylogeny. However, all methods of phylogenetic reconstruction make either implicit or explicit assumptions about the dynamics of nucleotide sequences, which, if violated, may result in misleading phylogenetic interpretation. Prior to commencing phylogenetic analysis therefore, it is essential that the modes of sequence evolution are taken into account in order to reduce the chance of confounding the "true" phylogeny (Sober, 1993; Yang *et al.*, 1994; Sanderson, 1995; Blouin *et al.*, 1998; Page and Holmes, 1998; Malhotra and Thorpe, 2000a).

2.1.1. Preliminary analysis of mtDNA sequence data

2.1.1.i *Sample size*

The use of single individuals to represent each taxon makes the implicit assumption that polymorphism does not affect phylogenetic analysis, because within group variation is negligible (Miyamoto and Cracraft, 1991). However, several

mitochondrial haplotypes may be identified from one locality (e.g. Pook *et al.*, 2000), therefore an adequate sample should be included to account for intralocality variation (Avise *et al.*, 1987).

2.1.1.ii *Base frequency*

Several studies report base frequency bias, being either AT (e.g., nematodes, Blouin *et al.*, 1998) or GC (e.g., mammals, Irwin *et al.*, 1991) rich according to the organism of study, and similar organisms are predicted to show similar base frequency patterns to each other (Swofford *et al.*, 1996; Page and Holmes, 1998). Animal mtDNA is also noted for an extreme bias in base composition at silent sites. Third codon positions are more likely to be silent than first or second, and if silent sites are to be used as phylogenetic markers there should be little or no change in bias between the taxa being compared (Irwin *et al.*, 1991). Transition bias should also be assessed (Aquadro *et al.*, 1984; Reeder, 1995; Wakeley, 1996) since transitions are generally more common that transversions (Li and Graur, 1991). As sequence divergence increases, the transition : transversion ratio decreases because of multiple substitutions at single transition sites. It is often assumed that transitions are reaching saturation at a ratio of 1.0 (ti:tv = 1:1), although complete saturation is not considered to have occurred until the ratio is about 0.5 (ti:tv = 1:2) (Holmquist, 1983; Mindell and Honeycutt, 1990; Reeder, 1995).

2.1.1.iii *Nucleotide substitution rate*

Felsenstein's (1981) assumption of constancy of substitution rates over all nucleotide sites is considered to be unrealistic for gene sequences coding products with biological functions (Yang *et al.*, 1994). Rate heterogeneity among bases is now accounted for in the more recent likelihood models (e.g., Tamura, 1994). Another common assumption is homogeneity of rates of substitution (evolution) between lineages (Tourasse and Li, 1999).

2.1.1.iv *Neutral evolution*

A highly controversial, yet important evolutionary hypothesis is that mitochondrial DNA undergoes neutral evolution. The neutral theory (Kimura, 1968) proposes that the vast majority of mutations are non-deleterious, having little or no effect on an organism's fitness (they are redundant with respect to physiology and function), and that the evolutionary dynamics of these mutations are governed solely by genetic drift. Only a fraction of mutations are strongly deleterious and these are quickly eliminated from populations by natural selection (King and Jukes, 1969; Akashi, 1999). Evidence conflicting with neutral theory has been noted in mitochondrial studies (e.g. Ballard and Kreitman, 1994; Ballard and Kreitman, 1995; Marjoram and Donnelly, 1994; Rogers and Harpending, 1992; Excoffier, 1990). However, most methods of phylogenetic reconstruction do assume neutral evolution of mtDNA, and therefore statistical tests of neutrality are a necessary supplement to any mtDNA analyses.

2.1.1.v *Pseudogenes*

Several studies of different vertebrates report the discovery of pseudogenes (gene duplications or inactivated genes) in the nuclear genome that are similar in sequence to the mitochondrial gene being targeted (Zevering *et al.*, 1991; Lopez *et al.*, 1994; Arctander, 1995; Sorenson and Fleischer, 1996; Zhang and Hewitt, 1996). Although the unwitting inclusion of "impostor" nuclear copies in phylogenetic analyses may introduce error (Sorenson and Fleischer, 1996), known nuclear copies may represent useful extinct ('fossil') mtDNA haplotypes that could be valuable for reconstructing ancestral states or outgroups in phylogenetic analyses, in situations where the selection of an extant outgroup is made difficult by high levels of divergence (Zischler *et al.*, 1995).

2.1.1.vi *Saturation and homoplasy*

Saturation results after so many base changes have occurred from recurrent mutation in the DNA sequence (and recombination, in the case of nuclear markers) that it is no longer possible to determine the history of state changes of a particular character, or nucleotide (Brower *et al.*, 1996; Smouse, 1998; Yang, 1998). The incidence of homoplasious characters, or characters identical in state but not identical by descent (Avise *et al.*, 1987; Smouse, 1998), therefore increases significantly with saturation. Hillis (1991) and Hillis and Huelsenbeck (1992) refer to such obscuring of phylogenetic signal as random noise: rates of change between nodes of a phylogenetic tree are high enough to effectively randomise the character state with respect to phylogenetic history.

The implementation of *a priori* differential weighting of molecular characters is often used to compensate for homoplasy in phylogenetic analyses (e.g., Irwin *et al.*, 1991; Mindell, 1991; Knight *et al.*, 1993; Hillis *et al.*, 1994; Yang, 1998), for instance by downweighting rapidly changing substitution sites, and upweighting slowly changing sites (Allard and Carpenter, 1996; Philippe *et al.*, 1996; Yang, 1998). The selection of an appropriate weighting scheme, however, is highly subjective (e.g., Philippe *et al.*, 1996; Milinkovitch *et al.*, 1996). A preferable alternative to the implementation of weighting schemes is to include a representative range of phylogenetic variation within a phylogeny by thorough taxonomic sampling, thereby cutting long branches and effectively reducing homoplasy without weighting (Allard and Carpenter, 1996; Milinkovitch *et al.*, 1996).

2.1.1.vii *Other causes of data incongruence*

Lineage sorting may explain incongruence between the gene tree and the species tree. Polymorphic alleles are either lost or 'shift' clade because the haplotypes are not

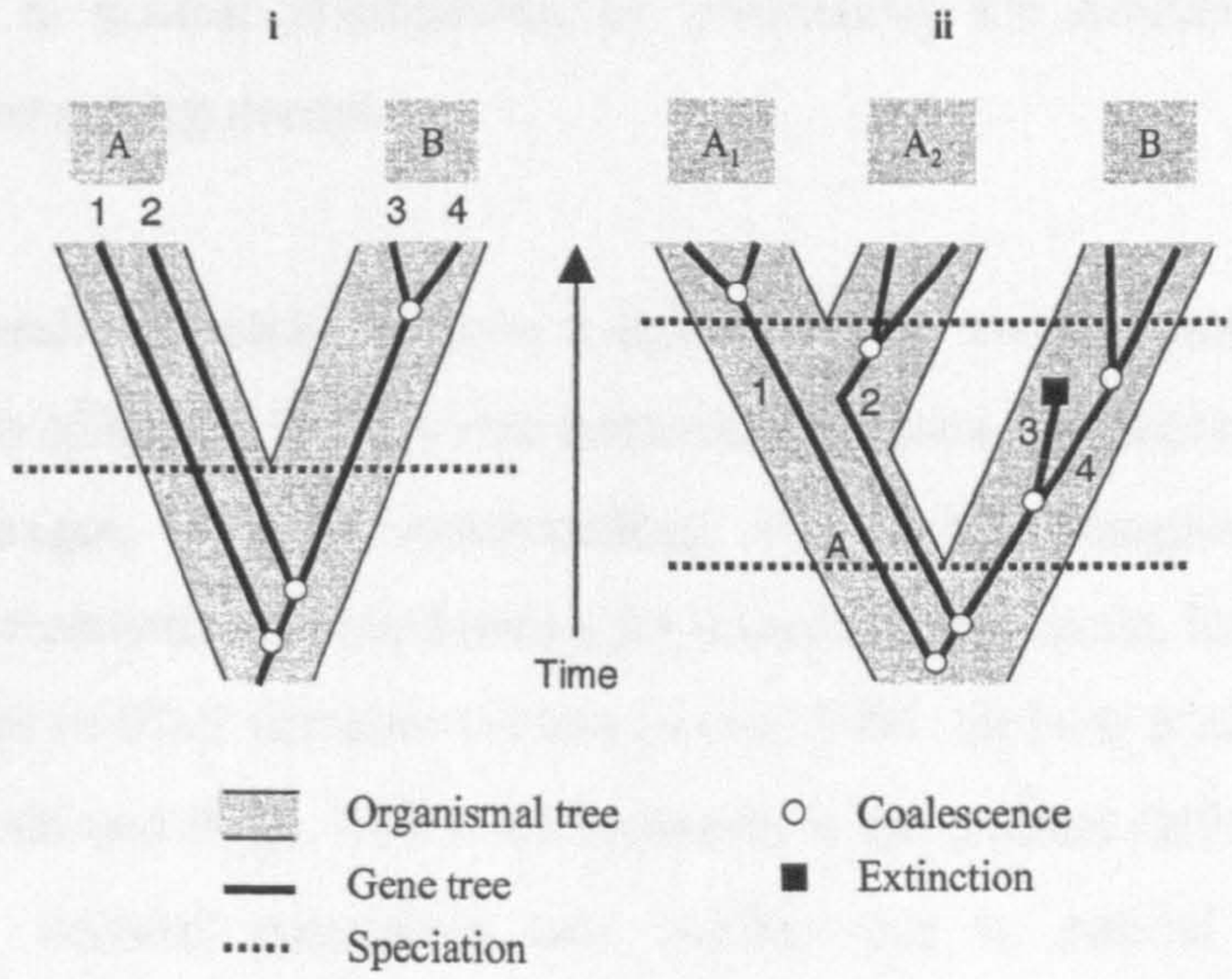


Figure 2.1. Lineage sorting (modified from Page and Holmes, 1998)

i) Illustrates a gene tree for four alleles (1-4) in two organismal lineages (A and B). Alleles 3 and 4 encounter their most common ancestor (coalesce) within lineage B, but coalescence in alleles 1 and 2 predates lineage A. Note particularly that alleles 1 and 2 do not form a monophyletic group - 2 is more closely related to 3 and 4 than to 1 even though 1 and 2 are found in the same species.

ii) Depicts a possible future scenario involving the same alleles and organismal lineages. Species A diverges to form species A_1 and A_2. Alleles 1 and 2 present in A when it speciated were inherited by A_1 and A_2 respectively, while allele 3 has become extinct.

equally shared or passed on to subsequent daughter populations, prior to later recognition of those clades as species or discrete taxonomic units. In other words the coalescence time for a given set of polymorphic alleles pre-dates the age of the species lineage (figure 2.1) (Moore, 1995; Page and Holmes, 1998). Nuclear genes appear to be more prone to lineage sorting (Moore, 1995), and it is generally proposed that incongruent sorting events could be identified from comparisons of several nuclear gene phylogenies, given that nuclear genes sort independently, unlike mtDNA genes which form a single linkage unit (Felsenstein, 1988; Miyamoto and Cracraft, 1991; Johnson and Clayton, 2000). The method gene tree parsimony (Maddison, 1997; Slowinski and Page, 1999) has been proposed as an alternative approach to phylogenetic reconstruction which accounts for the potential effects of lineage sorting

with respect to nuclear phylogenies, by minimising the number of required gene duplications or sorting events.

DNA is generally assumed to show a unidirectional, vertical pattern of inheritance, from parent to offspring. In very rare instances, however, horizontal transfer can occur between lineages, without interbreeding, via mobile fragments of DNA, or transposable elements. The mechanism for transfer is unknown, but may be mediated by retroviruses or other parasitic vectors (Avise, 1991; Brower *et al.*, 1996; Maddison, 1997; Slowinski and Page, 1999). Introgression is the gradual diffusion of genes from a previously isolated population into another due to natural hybridisation and differential gene flow between them. Signs of introgression include the discovery of particular "foreign" characters (e.g. alleles or mitochondrial haplotypes) against a mostly "normal" genetic or morphological background (Brower *et al.*, 1996).

2.1.1.viii *Metric data*

Distance measures used in phylogenetic reconstruction should be metric, and additive. The most similar sequences can be assumed to be the most closely related, and evolving at a constant rate, if the metric distances are also ultrametric. A metric is also ultrametric if, in a comparison of distances between three sequences, the two largest distances are equal. The distance measures are additive if the data satisfies a four-point condition $d(a,b) + d(c,d) \leq \text{maximum } (d(a,c) + d(b,d), d(a,d) + d(b,c))$ (see Page and Holmes, 1998).

2.1.1.ix *Combining data sets*

Opinions vary whether different data sets collected from the same taxa should be combined for analysis, analysed separately, or analysed separately before combining the independent estimates using consensus methods (see Hillis, 1987; Kluge, 1989;

Bull *et al.*, 1993). Combining data is considered appropriate if the various data sets are not significantly heterogeneous with respect to the reconstruction model (Bull *et al.*, 1993). Regarding mitochondrial genes, the view is held that combining genes should not adversely affect phylogeny retrieval, since mtDNA phylogenies from several genes do not constitute independent evidence of organismal phylogeny, as they are inherited as a single linkage unit. Sequencing an increasing number of genes will simply increase the number of available variable sites, and thereby increase the chance of obtaining the correct gene phylogeny for the entire mtDNA molecule (Cummings *et al.*, 1995).

2.1.2. Phylogenetic reconstruction

Many alternative methods of phylogenetic reconstruction exist, which are categorised according to the type of data used. Distance methods estimate phylogeny from distance measures calculated from the nucleotide data (e.g. UPGMA, neighbor joining, and minimum evolution). Discrete character methods (e.g. maximum parsimony and maximum likelihood) use individual characters such as nucleotide or amino acid sites. UPGMA and NJ implement clustering algorithms, whereby a specific sequence of algorithmic steps are used to determine a tree. Phylogeny-retrieval using MP, ML or ME, is based on predefined optimality criteria, often resulting several trees which are compared using a tree searching algorithm to determine which is the "best estimate" of phylogeny (Nei, 1991; Swofford *et al.*, 1996; Page and Holmes, 1998).

2.1.2.i *Distance methods*

Distance methods are based on the principle that evolutionary history could be reconstructed from metric distances between sequences (Page and Holmes, 1998), and use pairwise distance matrices converted from aligned DNA sequences as the input data. Two categories of distance methods are recognised. The first includes clustering

methods which seek the tree that best accounts for the 'observed' (pairwise sequence), distances (e.g. UPGMA and neighbor joining, Saitou, 1987) while the second group the minimum evolution methods, use optimality crietia to seek the tree whose sum of branch lengths is minimum. Distance algorithms are computationally fast, and account for the fact that, in reality, distances are rarely completely metric (Page and Holmes, 1998). The main drawbacks, however, are that information and sensitivity to saturation are lost during conversion of sequence data to distances, and there is a loss of flexibility to examine the deep structure of the data underlying the results (Cracraft and Helm-Bychowski, 1991). Secondly clustering algorithms generate only a single tree, which may not necessarily represent the tree that best fits the data (the correct tree), although see also Nei's (1991) comments regarding robustness of trees and recommended criteria for selecting the appropriate distance measure. In this respect alternative evolutionary hypotheses are not tested, and to be of any value the resulting tree requires a goodness of fit measure, between the observed distances obtained directly from the sequences themselves and the distances obtained from the tree (Nei, 1991; Page and Holmes, 1998).

2.1.2.ii *Discrete character methods*

The most widely used discrete character methods are maximum parsimony (MP) and maximum likelihood (ML), which choose among all possible trees using optimality criteria. A criterion is predefined for evaluating a tree, then a specific algorithm used to compute the value of the optimality criterion, followed by search for trees with the best values according to this criterion (Swofford *et al.*, 1996). Unlike clustering algorithms, optimality methods allow assessment of the quality of different trees, and competing evolutionary hypotheses can be tested against the data. These methods are NP-complete, in that there is no algorithm guaranteed to give the optimal tree, except by looking at all possible trees using exhaustive or branch-and bound searching. Consequently, tree searching becomes computationally expensive beyond the inclusion of 20 or more taxa (in the case of branch and bound), and calculation of all

trees becomes impossible, irrespective of the type of data being used (Page and Holmes, 1998). A solution to this problem is the use of heuristic searches for the optimal tree, in which subsets of potential trees are assessed, in the hope that the optimal tree may be revealed from one of the subsets (Swofford *et al.*, 1996; Page and Holmes, 1998). However, finding the optimum tree in any subset of a heuristic search is not guaranteed. The optimality principle may produce incorrect topologies when the number of characters is small, but works well if a sufficient number of nucleotides are examined (Nei *et al.*, 1998). Moreover, a presumed correct topology cannot be considered trustworthy unless the estimates of all or most interior branch lengths are significantly greater than 0. In this respect, testing the statistical reliability of an estimated tree, using an interior branch test (Rzhetsky and Nei, 1992) or bootstrap (Felsenstein, 1985b) may be preferable to finding the optimal tree (Nei *et al.*, 1998).

2.1.2.i.a Maximum parsimony

Maximum parsimony is based on the implicit assumption that evolutionary change is rare, in the sense that the data are not saturated, hence the tree that minimises the assumptions of change (the shortest tree) is considered to be the best estimate of the actual phylogeny (Hillis *et al.*, 1990). Parsimony relies on homologous, synapomorphic characters as being phylogenetically informative, and thereby the similarity that can be attributed to common ancestry is maximised (Hillis *et al.*, 1990; Swofford *et al.*, 1996). Parsimony cannot detect high levels of homoplasy, since it is assumed that homoplasy occurs at levels that do not interfere with phylogenetic inference (Hillis *et al.*, 1990). Therefore an accurate phylogeny will only be reconstructed from sequences with a low number of changes per nucleotide site (Felsenstein, 1978). In cases of homoplasy, there is also the tendency for longer branches to be put together ("long branch attraction") if they contain a sufficiently greater number of substitutions, irrespective of the true relatedness between those branches (Felsenstein, 1988). Implementing weighting schemes may compensate for excessive homoplasy (see Hillis *et al.*, 1990 for a summary), although opinions vary

whether weighting is beneficial (see Cracraft and Helm-Bychowski, 1991; Milinkovitch *et al.*, 1996; Philippe *et al.*, 1996; Vidal and Lecointre, 1998; Malhotra and Thorpe, 2000a).

2.1.2.i.b Maximum likelihood

The optimality criterion of maximum likelihood is the selection of the topology (phylogenetic hypothesis) that maximises the probability (likelihood) of observing a given data set (Huelsenbeck and Crandall, 1997; Page and Holmes, 1998; Swofford *et al.*, 1996; Nei *et al.*, 1998). First an evolutionary model is defined, taking into consideration the nature of evolution of individual nucleotide sites. Next the log-likelihood of having these nucleotides is computed for a given topology using a particular probability model (Felsenstein, 1981; Nei, 1991). Phylogenies are then inferred by seeking those trees with the highest likelihoods (Swofford *et al.*, 1996).

A major advantage of maximum likelihood is that the method presents a myriad of variations of phylogenetic estimation via explicit underlying models of character transformation, in contrast to the implicit assumptions of parsimony (Felsenstein, 1978; Brower *et al.*, 1996; Huelsenbeck and Crandall, 1997). In this respect likelihood offers the opportunity to compare a range of alternative evolutionary hypotheses. Violations of the underlying assumptions may lead to retrieving an incorrect phylogeny, and therefore the importance of selecting the correct model which reflects the actual evolutionary process cannot be understated (Yang *et al.*, 1994; Huelsenbeck and Crandall, 1997; Nei *et al.*, 1998). Of all methods of phylogenetic inference, maximum likelihood is probably least affected by sampling error, and does not suffer the inconsistencies experienced in parsimony, such as long branches attracting (Page and Holmes, 1998).

2.1.3. Branch support and tree stability

2.1.3.i *Support indices*

Indices of tree support include the consistency index (CI: Kluge, 1969), a measure of homoplasy in the data (the minimum number of steps required by the data divided by the number of steps in the most parsimonious tree) (Philippe *et al.*, 1996), and the retention index (RI: Farris, 1989), which reflects the fit of the characters on the most parsimonious tree (Lee, 1999). A similar principle is the total support index, which is the total support for the tree (the sum of the support indices for each clade in the tree - see below) divided by the length of the most parsimonious tree (Bremer, 1994). Indices only give overall indices of tree support, however, and do not determine which parts of the tree are robustly supported and which are not. Indices such as CI also vary depending on the number of taxa, so that CIs are not directly comparable across trees with different numbers of taxa (Philippe *et al.*, 1996).

2.1.3.ii *Wilcoxon signed-ranks test*

A non-parametric two-tailed Wilcoxon signed-ranks test is frequently used to test whether differences in tree length between the optimal tree and alternative topologies are explainable by chance alone (Templeton, 1983). This test is particularly useful for comparing *a priori* phylogenetic hypotheses against the optimal most parsimonious topology (e.g., Pook *et al.*, 2000).

2.1.3.iii *Kishino-Hasegawa and Shimodaira-Hasegawa tests*

The Kishino-Hasegawa (KH) test (Hasegawa and Kishino, 1989) is a parametric test for significant difference in tree length, although this test requires the assumption that all nucleotide sites are independently and identically distributed. The KH-test is

frequently used to compare likelihood trees, in which case the significance of a difference in log-likelihood scores is estimated. A recent review (Goldman *et al.*, 2000) claims that the KH-test is only appropriate when the topologies being compared are specified *a priori* (i.e. hypotheses derived independently of the data at hand), and should not therefore, be used to test optimal ML topologies against competing hypotheses. Two new tests are recommended for this purpose, the parametric SOWH-test (Goldman *et al.*, 2000) and the non-parametric Shimodaira-Hasegawa test (Shimodaira and Hasegawa, 1999).

2.1.3.iv *Bremer support*

One method of testing internal branch support is to calculate decay or support indices for each branch. A commonly used approach, Bremer support (Bremer, 1994), calculates the number of extra steps required to lose a branch in the consensus tree of near-most-parsimonious trees. A value, the branch support index (Bremer, 1994), is assigned to each branch in the tree. No guidelines exist advising what value constitutes a well supported group (Swofford *et al.*, 1996), but support indices do allow a qualitative assessment to be made, where the values assigned to each branch within a particular phylogeny can be gauged against each other.

2.1.3.v *Bootstrap*

The bootstrap involves creating a new data set from the original by randomly sampling the available characters with replacement. A phylogeny is reconstructed from the resampled data set, and the frequency at which a node reappears among different permutations is taken as a measure of its reliability or stability (Hillis *et al.*, 1990). Bootstrap can be applied to distance and discrete methods. Consequently the underlying mathematical theory has been thoroughly scrutinised (e.g., Zharkikh and Li, 1992; Hillis and Bull, 1993; Sitnikova *et al.*, 1995; Zharkikh and Li, 1995), and the

reliability or appropriateness of bootstrap in systematics has been questioned and heavily criticised (for a critique see Sanderson, 1995).

The problem of applying reliable confidence limits to phylogenies may be overcome using alternative methods, such as nested clade analysis (Crandall, 1994; Templeton *et al.*, 1995; Templeton and Sing, 1995; Templeton, 1998). 95% confidence limits are applied in a preliminary calculation prior to the construction of a single, minimum spanning tree, which illustrates all most parsimonious solutions (Creer, 2000). This method, designed specifically for intraspecific phylogenetic reconstruction, establishes the probability of obtaining a "non-parsimonious" inference from mitochondrial or nuclear DNA data sets and may offer insights to the various causal factors responsible for the pattern observed (Brower *et al.*, 1996).

2.1.4. Molecular clock calibrations in squamates

The molecular clock hypothesis of Zuckerkandl and Pauling (1965) postulates that the rate of molecular evolution is constant over time among evolutionary lineages, therefore two nucleotide sequences accumulate the same number of substitutions since their divergence from a common ancestral sequence (Tourasse and Li, 1999). Assuming a provisional molecular clock provides a useful guide for estimating species duration (Walker and Avise, 1998), for timing divergence (e.g., Thorpe *et al.*, 1994), and for estimating population distribution range shifts (Riddle, 1995). Consequently, clock assumptions must be treated cautiously, since the differences in mtDNA evolution between higher vertebrate groups have not yet been fully identified, and many studies have shown considerable rate heterogeneity (Hasegawa *et al.*, 1985; Martin *et al.*, 1992; Rand, 1994; Hillis *et al.*, 1996; Mindell *et al.*, 1996). Nevertheless, the use of clocks for closely related taxa is generally considered to be more reliable than for distantly related taxa (Caccone *et al.*, 1997), since rates of evolution of a particular gene are likely to be stable in closely related taxonomic groups, with similar

life histories, metabolic rates and generation times (see also Brown *et al.*, 1979; Martin *et al.*, 1992). In this respect, the estimation of "local" rates for closely related taxa may be preferable over a "universal" rate (e.g. Hillis *et al.*, 1996). Estimations can also be complicated by the variation in rates of evolution of different sections of the mitochondrial DNA molecule. Rates of overall sequence divergence estimated for the entire molecule (e.g., from RFLP studies) may thus be misleading for studies using sequences from specific genes. In addition, clock calibration requires a knowledge of the timing of certain geologic events. However, these estimates will always be very approximate, since most geological events took place over prolonged periods of time. Fossil evidence may be useful if reliably dated.

So far, there have been no specific estimates for the rate of sequence evolution of the cytochrome *b* and ND4 genes in squamate reptiles. Zamudio and Greene (1997) calculated a "ballpark" estimate of 0.47-1.32 % My^{-1} for overall mtDNA divergence rates for small to medium-sized ectotherms. However these estimates were calculated from the entire mitochondrial DNA genome, based on RFLP data, or several mitochondrial genes with differential rates of nucleotide substitution (in particular 12S and 16S which are very slow). Consequently these estimates are difficult to relate to sequence variation in specific mitochondrial genes. However, Zamudio and Greene's (1997) estimated rates of cytochrome *b* and ND4 sequence differentiation together with the distribution of mtDNA haplotypes in bushmasters (*Lachesis*) appear to coincide well with the timing of putative vicariant geologic events, the uplifting of the Andes (14-11 Ma) and the Cordillera de Talamanca (8-5 Ma). Accepting these events as causing the split between Central and South American bushmasters (8.44% sequence divergence) and between *Lachesis melanocephala* and *L. stenophrys* (5.30% sequence divergence) respectively leads to estimates of divergence rates of 0.60-0.76 % My^{-1} and 0.66-1.06 % My^{-1} (Pook *et al.*, 2000). These estimates present a narrower range of sequence divergence for reptiles, and remains slower than estimates for endotherms.

(Wüster *et al.*, in press) present an alternative calibration of 1.09-1.77 % My^{-1}. This estimate is based on the final emergence of the Isthmus of Panamá 3.5 Ma (Coates and Obando, 1996), and the mean sequence divergence of combined cytochrome *b* and ND4 (total: 1388 base pairs) among three sequences of South American *Porthidium*. Although higher than the previous estimates of squamate mtDNA molecular clocks, this new estimate seems realistic based on the information available. *Porthidium* is a poor overwater disperser (the genus does not occur on any islands except one continental shelf island periodically connected to the mainland during the Pleistocene), therefore the arrival of *Porthidium* in South America is likely to have coincided with the emergence of the Panamá Isthmus. If this assumption is correct, the emergence if the Isthmus would represent the earliest possible time of divergence of *Porthidium* mitochondrial haplotypes in South America.

2.1.5. Phylogeny of the *Crotalus viridis* species complex

The limitations encountered though comparison of morphological characters alone (Hillis, 1987; Miyamoto and Cracraft, 1991), and the controversy surrounding use of the subspecies category (Wilson and Brown, 1953; Thorpe, 1987; Frost and Hillis, 1990; Pook *et al.*, in press), emphasises the need for a robust molecular phylogeny of *C. viridis* for a fuller understanding of the geographic variation in this species complex.

2.1.6. Aim

In this chapter, the phylogenetic relationships of the *C. viridis* complex are reconstructed based on mitochondrial cytochrome *b* and ND4 gene sequence data. The intraspecific taxonomic associations are evaluated, and discussed based on an historical interpretation of the results. The phylogeny is tested for biogeographic congruence against the phylogenies of other western U.S. polytypic species, *Pituophis*

catenifer and *Phrynosoma douglasi*. Hypotheses of subspecies monophyly, origin of the Mojave toxin-like phospholipase (Concolor toxin) in the venom of adult *C. v. concolor*, and evolution of small body size in the *Crotalus viridis* species complex, are also tested against the resulting phylogeny. A biogeographic interpretation of the results is presented.

2.2 Materials and methods

Sixty-eight individuals representing 39 US county localities across the range of *Crotalus viridis*, and 1 locality in Mexico (figure 2.2) are used (see Appendix I for sample details). Blood samples were taken from live snakes by caudal venepuncture, or other tissues (muscle, heart, liver, skin) were obtained from institutional collections. Blood was stored in a cell lysis buffer (2.0% SDS; 100mM Tris pH 8.0; 0.1M EDTA pH 8.0), and other tissue-types in 90% ethanol. Template DNA was prepared using a standard Proteinase K protocol (Buffone, 1985; Miller *et al.*, 1988; Sambrook *et al.*, 1989). Mitochondrial cytochrome *b* (758 bp) and ND4 (900 bp) fragments were amplified by double-stranded PCR (Saiki, 1988). The cyt *b* primers were 5'-TCA AAC ATC TCA ACC TGA TGA AA-3' (L-strand - modified from (Kocher *et al.*, 1989) and 5'-GGC AAA TAG GAA GTA TCA TTC TG-3' (H-strand, modified version of primer MVZ 16 of (Moritz *et al.*, 1992). The 5' ends of these primers correspond to positions 14977 and 15735 of the total mtDNA sequence of *Dinodon semicarinatus* (Kumazawa *et al.*, 1998), whereas the ND4 primers were primers ND4 and Leu of (Arévalo *et al.*, 1994). All PCR products were purified using a GenElute™ (Supelco) nucleic acid purification kit. Detailed protocols for DNA template preparation, PCR reaction conditions, and PCR amplified product purification are provided in Appendix II. The cyt *b* and ND4 fragments were sequenced by automated, single-stranded DNA sequencing on an ABI 377 DNA Sequencer, following the protocol of the manufacturer (Appendix II). Resulting sequence chromatograms were viewed in Chromas 1.51 (Technelysium Pty Ltd.), and the corresponding text sequences checked carefully and aligned by eye in a simple text editing program.

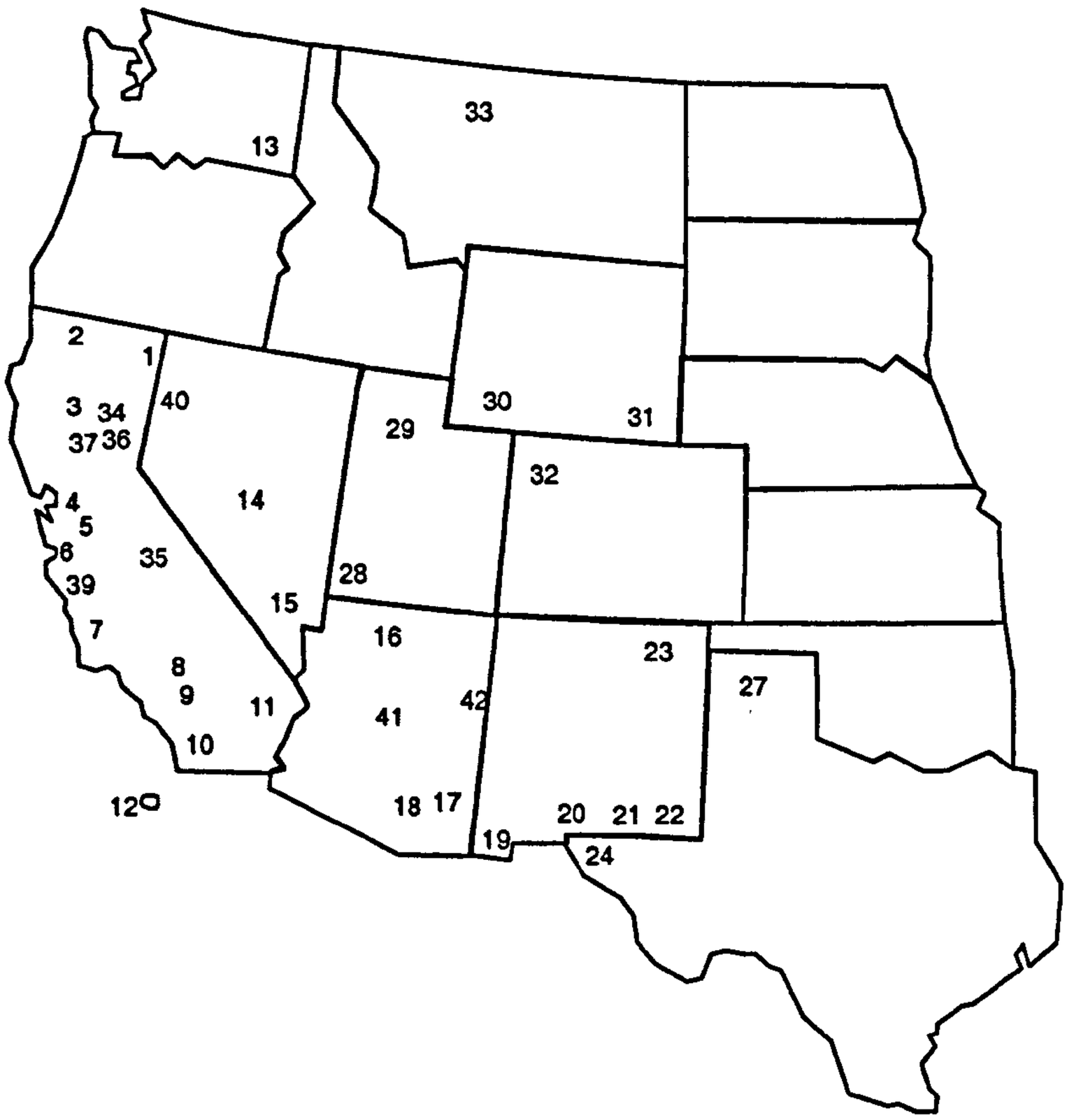
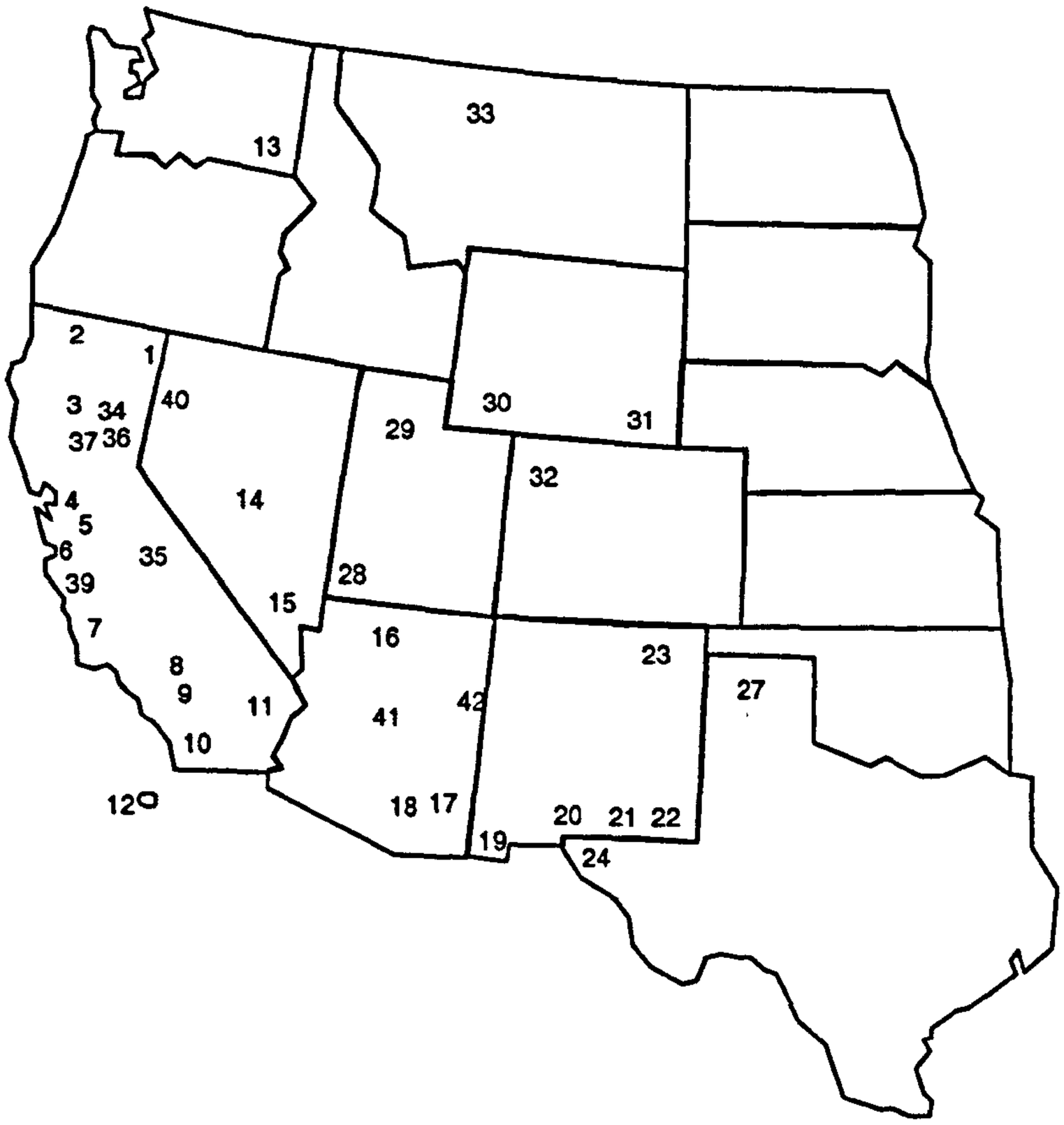


Figure 2.2. Sampling localities (counties) for *C. viridis* in the western USA and Mexico (**Note:** Locality numbers follow those of Pook *et al.*, 2000; see fig 1.10 for state labels). Localities not used in the present phylogeny have been removed, therefore, not all numbers follow sequentially): **UNITED STATES California**: 1. Modoc; 2. Siskiyou 3. Tehama; 4. Alameda; 5. Stanislaus; 6. Santa Cruz; 7. San Luis Obispo; 8. Los Angeles; 9. San Bernardino; 10. San Diego; 11. Riverside; 34. Plumas; 35. Tulare; 36. Sierra; 37. Butte; 39. Monterey **Washington:** 13. Whitman; **Nevada:** 14. Nye; 15. Clark; 40. Washoe **Arizona:** 16. Coconino; 17: Graham; 18. Pima, 41. Gila; 42. Apache; **New Mexico:** 19. Hidalgo; 20. Dona Ana; 21. Otero; 22. Eddy; 23. Colfax; **Texas:** 24. El Paso; 27. Sherman; **Utah:** 28. Washington; 29. Salt Lake; **Wyoming:** 30. Sweetwater; 31. Laramie; **Colorado:** 32. Moffat; **Montana:** 33. Chouteau. **MEXICO** 12. South Coronado Island.



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2.2.1 Preliminary analyses

All analyses were carried out in PAUP* version 4.0b3a unless stated otherwise. Specimens with identical sequences were excluded from the analysis to avoid the computational problems encountered in maximum parsimony and maximum likelihood analysis with high numbers of OTUs (Milinkovitch *et al*, 1996).

2.2.1.i *Independent assessment of cytochrome* b *and ND4 genes*

In order to test the compatibility of the two sets of sequence data prior to a combined analysis, independent assessments were made of 678 bp cytochrome *b* and 652 bp ND4 mitochondrial fragments for 61 unique haplotypes of *Crotalus viridis*. *Crotalus scutulatus scutulatus* and *C. durissus terrificus* were included as outgroups.

The sequences were translated into amino acids to ensure that there were no stop codons, the absence of which provides some evidence that the sequences were mitochondrial and not nuclear pseudogenes (Lopez *et al.*, 1994; Arctander, 1995; Sorenson and Fleischer, 1996; Zhang and Hewitt, 1996) in MEGA 1.02 (Kumar *et al.*, 1996). Pseudogene sequences display various degrees of homology with their mitochondrial counterparts, and depending on the taxa and regions involved also show variation in divergence. Other affected characteristics include variation in codon position bias, transition-transversion ratio, and synonymous versus non-synonymous substitutions (Irwin *et al.*, 1991); Lopez *et al.*, 1994; Arctander, 1995; Sorenson and Fleischer, 1996; Zhang and Hewitt, 1996). Typical clues indicating the presence of pseudogenes include extra non-specific bands in the PCR, sequence ambiguities or persistence of background bands, unexpected insertions or deletions, frameshifts, or stop codons, radically different nucleotide sequences from those expected, and unusual or contradictory tree topology (Zhang and Hewitt, 1996).

Similarity in substitution rate was tested by plotting uncorrected pairwise divergences for both genes against each other and fitting a regression line through the points (figure 2.4). To determine the validity of combining genes, a statistical test for phylogenetic congruence was carried out using a partition-homogeneity test (Bull *et al.*, 1993; Johnson and Clayton, 2000), involving a heuristic search (100 replications, SPR branch swapping, random addition of sequences) with character partitions set for cytochrome *b* and ND4 respectively (Farris *et al.*, 1995; Bull *et al.*, 1993; Banford *et al.*, 1999; Johnson and Clayton, 2000).

Consensus phylogenies were generated from heuristic searches (random addition of sequences, TBR branch swapping, 100 replications), providing a qualitative assessment of topological similarity.

The two data sets were combined (taking care not to disrupt the codon reading frames) once it was established that there were no significant differences that could lead to erroneous phylogenetic interpretation (see results).

2.2.1.ii *Preliminary assessment of combined data set*

Sequences were compared for uniformity of base frequencies with a G-test of heterogeneity using rpgtest (Sokal and Rohlf, 1999). The null hypothesis predicts a homogeneous distribution of base frequencies across all OTUs, and any departure is explained by sampling error or scatter (Fowler and Cohen, 1993). The distribution of base frequencies across first, second, and third codon positions was also assessed, and the level of base frequency bias at each of the three codon positions was calculated according to equation (2.1), where C is the compositional bias and c_i is the frequency of the *i*th base (Irwin *et al.*, 1991).

Equation 2.1

$$C = (^2/_3)\sum_{i=1}^{4} |c_i - 0.25|$$

The McDonald and Kreitman test (McDonald and Kreitman, 1991; Ballard and Kreitman, 1994; Ballard and Kreitman, 1995) implemented in the DnaSP program (Rozas and Rozas, 1999) was used to test the neutrality of the data. This test involves the prediction, that in neutrally evolving genes the ratio of non-synonymous to synonymous fixed substitutions between species should be the same as the ratio of non-synonymous to synonymous polymorphisms within species. Sixty-one ingroup taxa (*Crotalus viridis*) were tested against four congeneric outgroup taxa (three *Crotalus durissus* and one *Crotalus scutulatus*).

Rate homogeneity was tested using three-species relative rate tests (Takezaki *et al.*, 1995), executed in PHYLTEST (Kumar, 1996), to test the hypothesis of rate constancy of nucleotide substitutions within the ingroup clades, and between the ingroup and outgroups. These tests compare the difference in number of substitutions (genetic distance) between two closely related taxa against a third, more distantly related, reference lineage (outgroup) (Tourasse and Li, 1999). Given that only two sister groups and one outgroup are compared at a time, the following combinations of known monophyletic clades within the *C. viridis* phylogeny (Pook *et al.*, 2000) were arranged accordingly (*ab = abyssus; ca = caliginis; co = concolor; ce = cerberus; he = helleri; lu = lutosus; or = oreganus; nu = nuntius; vi = viridis*):

1. (((*ab,ca,co,ce,he,lu,or*),(*nu,vi*)),*Crotalus scutulatus*)
2. (((*ce*),(*ab,ca,co,ce,he,lu,or*)),(*nu,vi*)
3. (((*ca,he,co*),(*lu,or*)),*ce*)

The hypothesis of overall rate constancy ("clockness") of the tree was also tested by conducting a Shimodaira-Haswegawa test (Shimodaira and Hasegawa, 1999) which compares the likelihood scores of an unconstrained tree and a tree in which a molecular clock model was enforced.

The shape of a tree-length distribution is thought to provide a good indication of the presence of phylogenetic signal in the data set (Hillis, 1991) and (Hillis and

Huelsenbeck, 1992). To evaluate the probability of phylogenetic signal in the combined data set, therefore, skewness (g_1) statistics were calculated from 10^6 randomly generated trees. Distributions with a strong left skew indicate strong phylogenetic signal, and hence a correlation among characters beyond that expected at random (Hillis, 1991; Hillis and Huelsenbeck, 1992). Levels of saturation for first, second, and third codon position transitions and transversions respectively, were also determined from isometric plots of uncorrected pairwise sequence divergences against Tamura-Nei (Tamura and Nei, 1993; Tamura, 1994) corrected pairwise divergences. Deviation from the isometric line represents a qualitative measure of degree of saturation (Zamudio and Greene, 1997, and references therein). Yang (1998) suggests that saturation only gives cause for concern at much higher levels of sequence divergence (30-40% overall uncorrected divergence).

2.2.2 Phylogenetic reconstruction

2.2.2.i *Parsimony*

The combined cytochrome *b* and ND4 data sets were subjected to unweighted parsimony analysis. Previous results revealed that differentially weighting codon positions, or downweighting transitions, neither improved resolution within the tree, nor affected composition of the clades (Pook *et al.*, 2000).

Trees were generated by a heuristic search specifying TBR (tree bisection-reconnection) branch-swapping, for 100 random addition sequence replicates. Branch support was tested using the bootstrap (Felsenstein, 1985b) with 100 iterations, NNI branch-swapping, and, in order to reduce search-time, the search was constrained to keeping the best 1000 trees only. Branch support indices (Bremer, 1994) were calculated for all internal branches of the tree, where the support index for a given branch is the length of the shortest consensus tree in which that branch collapses, minus the length of the most parsimonious tree (hence the number of steps

required to collapse that branch). Starting with the most parsimonious tree, heuristic searches are executed, using the 'keep' option in PAUP to retain trees one step longer in each successive search. A consensus tree is produced at the end of each search in order to calculate the support indices for each clade.

2.2.2.ii *Maximum likelihood analysis*

The computer program Modeltest (Posada and Crandall, 1998) was used to select the model of DNA sequence evolution most fitting to the *Crotalus viridis* sequence data. The sequence data file, and the Modeltest program command file, "modelblock", are executed consecutively in PAUP*. Then the program calculates likelihood scores corresponding to the specific data set for each one of 56 models of increasing complexity (see also Wiens *et al.*, 1999 for an alternative nested approach). The likelihood scores are next compared using the Modeltest program, which calculates which model best fits the data in a likelihood ratio test.

An heuristic search (starting with a neighbor joining tree) was carried out under the likelihood optimality criterion, and imposing the parameters provided by Modeltest. Given that the Modeltest parameters were initially based on a Jukes Cantor neighbor joining tree (i.e the most basic distance tree), likelihood scores were re-estimated from the new tree resulting from the first search (estimate rate matrix, base frequencies, proportion of invariant sites, shape) to test if the parameters could be changed to achieve an even better likelihood score. This procedure was repeated until the parameters, and hence the likelihood score, were consistent. The tree with ultimately the highest score was considered to be the best tree (the most likely phylogenetic interpretation of the data). A bootstrapped likelihood tree was also produced to assess branch support, from a heuristic search starting with a neighbor-joining tree, with SPR branch swapping for 100 replicates, and imposing the parameters which achieved the best tree.

2.2.2.iii *Tests of alternative phylogenetic hypotheses*

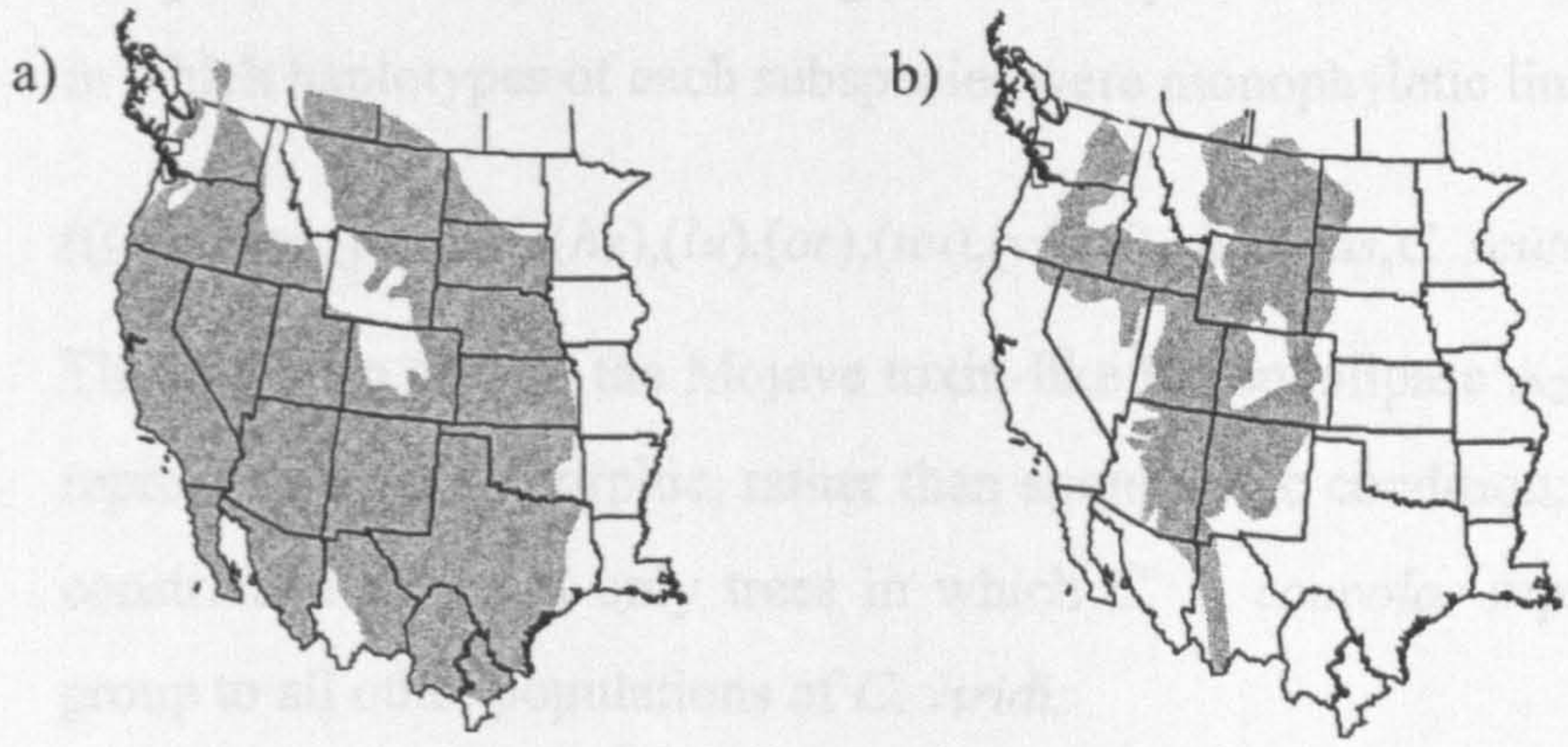


Figure 2.3. Distribution of a) *Pituophis catenifer* (gopher snake) and b) *Phrynosoma douglasi* (short-horned lizard) in the western United States, southern Canada and northern Mexico. See figure 1.10 for state/country labels.

A number of alternative phylogenetic and phylogeographic hypotheses were tested using two-tailed Wilcoxon signed-ranks tests (Templeton, 1983), which show whether the cladograms predicted by the alternative hypotheses differ significantly from the most parsimonious tree obtained, or whether differences in topology (tree length) are likely to have arisen by chance alone. Heuristic searches (random addition, TBR, 100 replications) were conducted, constraining each analysis to retain only the most parsimonious trees compatible with the hypothesis to be tested. Each of the constraint trees and a randomly selected subset of 100 of the most parsimonious trees were then compared in the Wilcoxon signed-ranks test for significant differences in tree length. The same hypotheses were then tested using the Shimodaira-Hasegawa test on maximum likelihood trees. The following hypotheses were tested (see Appendix III trees):

2.2.2.iii.a Hypotheses based on the Crotalus viridis phylogeny

i) The haplotypes of the nine conventional subspecies of *Crotalus viridis* form independent monophyletic lineages; the analysis was constrained to retain trees in which haplotypes of each subspecies were monophyletic lineages

(((*ab*),(*ca*),(*co*),(*ce*),(*he*),(*lu*),(*or*),(*nu*),(*vi*)),*C. durissus*,*C. scutulatus*)

ii) The ability to secrete the Mojave toxin-like Phospholipase A_2, Concolor toxin, represents a plesiomorphic, rather than apomorphic condition; the analysis was constrained to retain only trees in which *C. v. concolor* represents the sister group to all other populations of *C. viridis*

(((*ab*,*ca*,*ce*,*he*,*lu*,*or*,*nu*,*vi*,)*co*),*C. durissus*,*C. scutulatus*)

iii) Small body size arose only once in *C. viridis*; the analysis was constrained to retain only the most parsimonious tree in which *C. v. concolor* and *C. v. nuntius* are sister taxa

((*ab*,*ca*,*co*,*ce*,*he*,*lu*,*or*,*vi*,(*co*,*nu*)),*C. durissus*, *C. scutulatus*)

2.2.2.iii.b Phylogeographic hypotheses

The aim of the following tests was to determine whether the distributions of other western U.S. reptiles were shaped by the same historical events, by testing for congruence in phylogeographic pattern between *Crotalus viridis* and two other western North American species with similar ranges (figure 2.3), *Pituophis catenifer* (using locality and sequence information published in Rodríguez-Robles and de Jesús-Escobar, 2000) and *Phrynosoma douglasi* (Zamudio *et al.*, 1997). Phylogenetic congruence would predict that the three species phylogenies share similar biogeographical histories, whilst incongruence would suggest that the observed phylogeographic patterns under comparison have resulted from different underlying evolutionary events. Initially the analysis was carried out using the sequence data for

Crotalus viridis. The procedure was then repeated using the published sequences for *Pituophis catenifer* (Rodríguez-Robles and de Jesús-Escobar, 2000), but could not be repeated using the *Phrynosoma* sequences since not enough published information was available to match the specific haplotypes to their respective localities. Note that in each case, sequences without a corresponding locality in the constraint phylogeny have been excluded from the relevant analysis (e.g. haplotypes of *Phrynosoma* are not found in the southern Pacific region, hence when constraining *Crotalus viridis* to be consistent with *Phrynosoma* phylogeography, the southern Pacific haplotypes, *C. v. helleri*, were excluded). Consequently unconstrained tree lengths vary between analyses.

Abbreviations: SER = south and east of the Rocky Mountains; AZ = Arizona (Central Highlands only) PC-N = Pacific Coast north of the Sierra Madre Mountains; PC-S = Pacific Coast South of the Sierra Madre Mountains; GB = Great Basin.

iv) Congruence in phylogeographic pattern between *Crotalus viridis* and *Pituophis catenifer*:

a) *C. viridis* data constrained to be consistent with *Pituophis catenifer* phylogeography

(outgroup,(((SER),(AZ)),((PC-N), ((GB),(PC-S)))))

b) *Pituophis catenifer* constrained to be consistent with *Crotalus viridis* phylogeography

(outgroup,(SER,(AZ,(((PC-N),(GB)),(PC-S)))))

v) Congruence in phylogeographic pattern between *Crotalus viridis* and *Phrynosoma douglasi.*

C. viridis data constrained to be consistent with *Phrynosoma.*

(outgroup,(PC-N,((GB),(SER))))

vi) Congruence in phylogeographic pattern between *Pituophis catenifer* and *Phrynosoma douglasi*.

P. catenifer data constrained to be consistent with *Phrynosoma*.

(outgroup(PC-N(GB),(SER)))

2.2.2.iv *Molecular clock*

The modified *Lachesis* clock, 0.6-1.06 % My^{-1} (Pook *et al.*, 2000) and the *Porthidium* clock, 1.09-1.77 % My^{-1} (Wüster *et al.*, in press), were used to estimate the time of divergence (uncorrected p-distance divided by rate) of the two most basal clades in *Crotalus viridis*, including the separation of eastern and western populations, and the split of *C. v. cerberus* from other western taxa (Pook *et al.*, 2000).

2.3 RESULTS

2.3.1 Sequence information

61 unique haplotypes out of 68 samples of *Crotalus viridis* were obtained for cytochrome *b* sequence corresponding to the segment between positions 15045 and 15720 of the total mtDNA sequence of *Dinodon semicarinatus* (Kumazawa *et al.*, 1998), and ND4 sequence corresponding to the segment between positions 11743 and 12396 of *Dinodon*. Sequence details are summarised in Table 2.1. Uncorrected (p) sequence divergence ranged between 0.2 % and 6.1% among ingroup taxa, 7.1%-7.8% between *C. viridis* and *C. scutulatus*, and 12.7%-13.6% between *C. viridis* and *C. durissus*. The distance matrix for the combined data set is summarised in Table 2.2.

Table 2.1. Mitochondrial DNA sequence information relating to sequences used in phylogenetic reconstruction in *C. viridis*.

		Excluding outroups		Including outgroups	
	Total no. bp	No. variable sites (%)	No. parsimony informative sites (%)	No. variable sites (%)	No. parsimony informative sites (%)
Cyt *b*	678	95 (14.01)	73 (10.77)	150 (22.12)	85 (12.54)
ND4	669	109 (16.72)	83 (12.41)	172 (26.38)	93 (14.26)
Combined	1330	204 (15.33)	156 (11.73)	322 (24.21)	178 (13.38)

Table 2.2. The ranges of uncorrected pairwise sequence divergence within and between clades of *Crotalus viridis*. ab1 refers to the *C. v. abyssus – C. v. lutosus* clade; ab2 refers to the *C. v. abyssus – C. v. concolor* clade.

	lu	ab2	co	ca	he	or	ce	nu	vi
ab1	0.45-1.67	2.14	2.29-2.45	2.37	2.37-2.83	2.60-3.30	3.62-3.94	5.86-6.03	5.70-6.11
lu	0.08-1.90	2.29-2.60	2.45-2.83	2.52-2.76	2.37-2.91	2.06-2.83	3.38-4.17	5.78-6.35	5.62-6.43
ab2		-	0.23-0.45	1.98	1.83-2.45	2.21-3.30	3.46-3.78	5.78-5.86	5.54-5.94
co			0.08-0.45	2.06-2.14	1.83-2.60	2.14-2.99	3.46-3.94	5.78-6.03	5.54-6.11
ca				-	0.38-2.14	2.21-3.30	3.46-3.78	5.54-5.70	5.38-5.78
he					0.08-0.98	1.98-3.23	3.15-3.94	5.21-5.86	5.13-5.94
or						0.08-2.06	2.99-3.86	5.05-6.35	5.21-6.43
ce							0.15-0.91	5.54-5.78	5.30-6.11
nu								0.08-0.45	0.08-1.52
vi									0.15-1.44

2.3.2 Results of preliminary analyses

Independent assessment of cytochrome *b* and ND4 genes. Levels of sequence divergence for ND4 and cyt *b* were shown to be close (with a regression slope of 1.3; figure 2.4), suggesting that the rate of substitution (divergence) in both mitochondrial fragments are approximately comparable. These results were further corroborated by the partition-homogeneity test, which did not detect phylogenetic incongruence between the respective data sets (P=0.667). The resulting most parsimonious phylogenies for each fragment were poorly resolved in both instances. Clade composition was largely congruent, however, with the exception of *C. v. cerberus*,

which forms a sister lineage to all *C. viridis* in the cyt *b* tree, but a sister group to populations occurring west of the Rocky Mountains in the ND4 tree. Monophyly of *C. v. cerberus* was particularly weakly supported in the cytochrome *b* phylogeny, requiring only 1 step to collapse the clade, in contrast to the ND4 phylogeny in which the position of *C. v. cerberus* was strongly supported, needing 8 steps to collapse the branch. Topological congruence together with the similarity in the degree of resolution and substitution rate further substantiates evidence against the accidental amplification of nuclear copies.

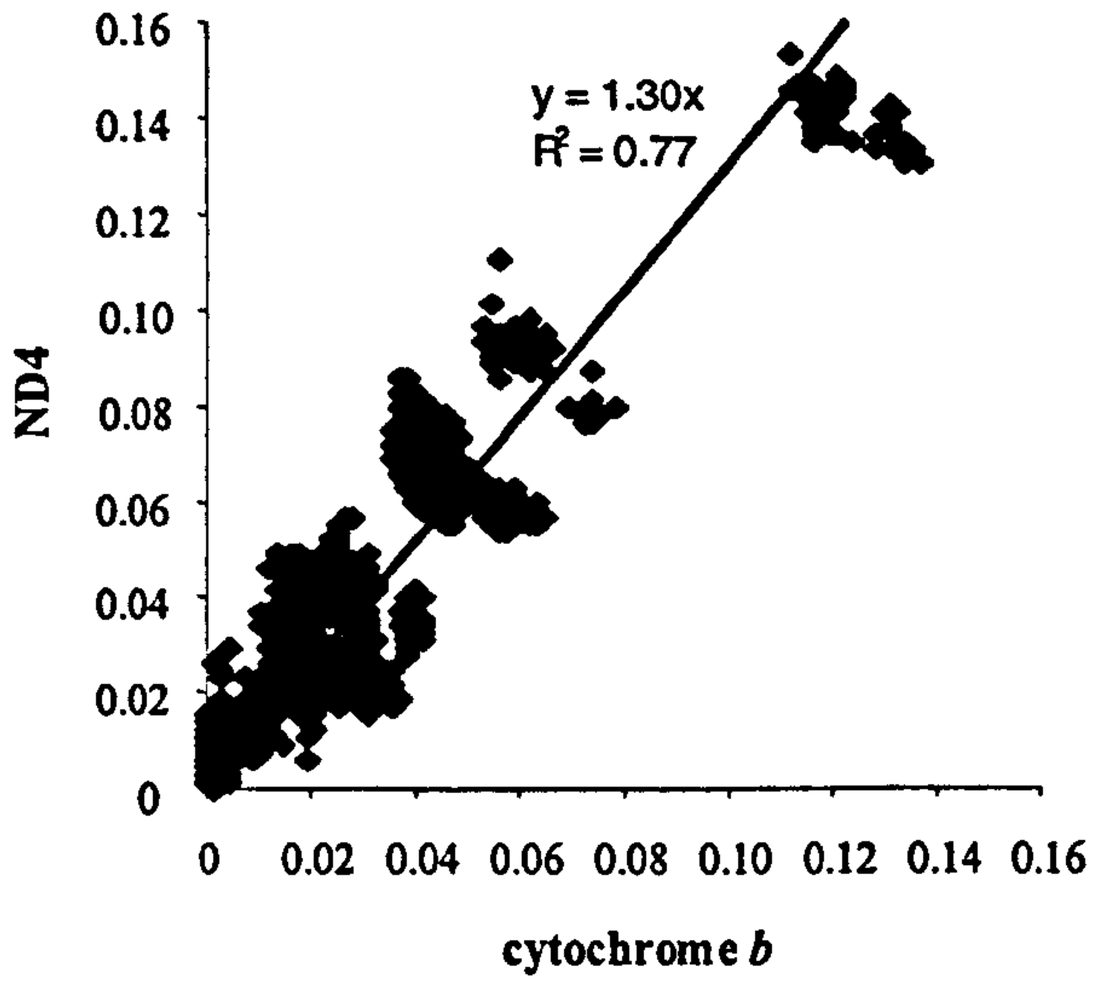


Figure 2.4. The relationship of percentage divergence of mitochondrial ND4 with Cyt *b* in *Crotalus viridis*. The regression slope is 1.3 suggesting relatively similar rates of base substitution between the two fragments. The evolutionary rate in ND4 is slightly faster.

2.3.2.i *Preliminary assessment of combined data set*

The results of the G-test failed to reject the null hypothesis of data homogeneity, confirming an even distribution of base frequencies across all OTUs ($G_C = 3.25$; $G_G = 6.12$; $G_A = 2.87$; $G_T = 3.5$; df = 60 $P>0.05$). Proportions of base frequencies across

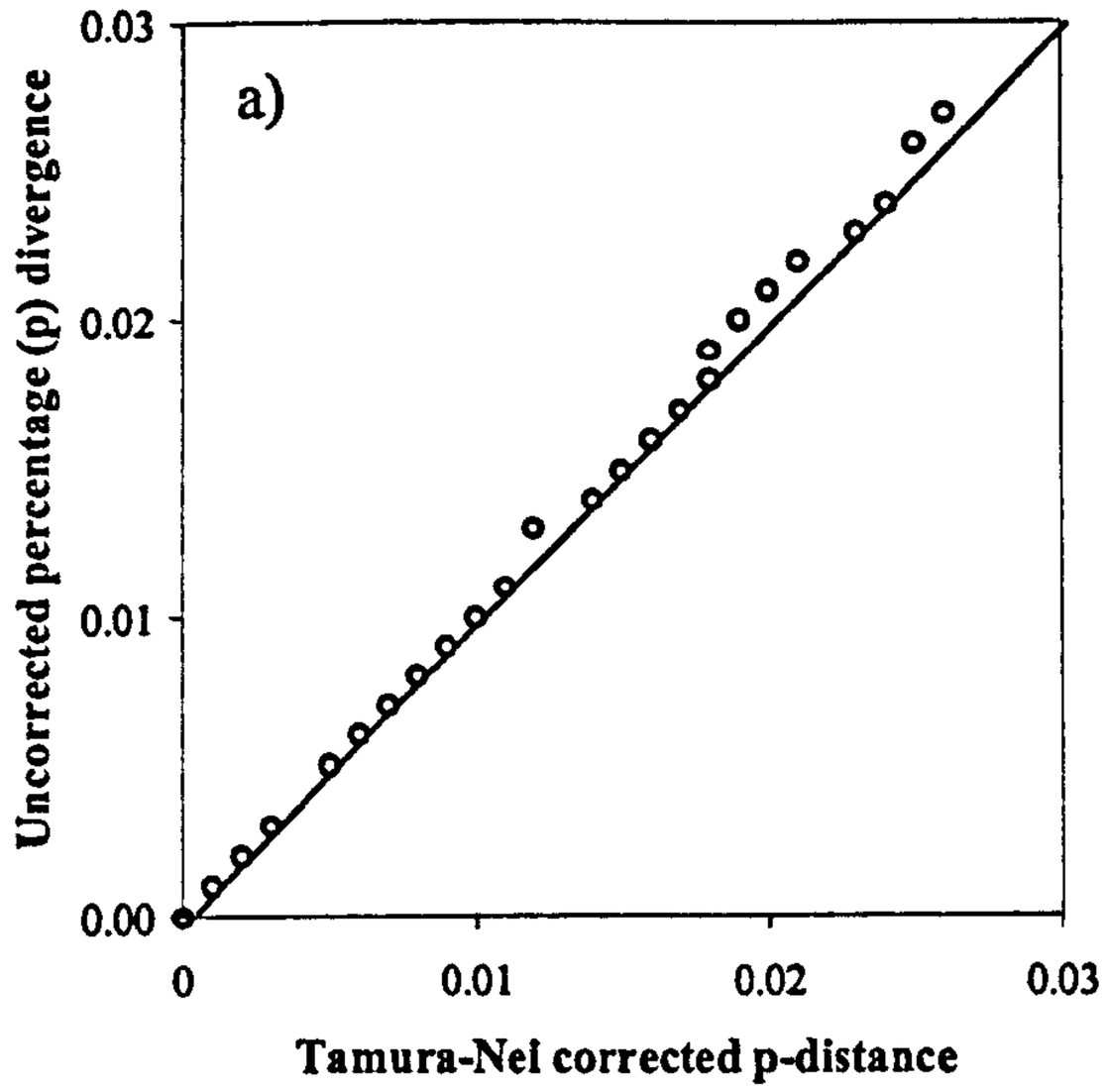
entire sequences were consistent for cyt *b* and ND4 respectively, and as expected the most variation in base frequency is seen at third codon positions, followed by first, and second showing the least (Table 2.3). The low values of codon bias suggest negligible bias in the data set, and show remarkable closeness to the values obtained by Irwin *et al.* (1991) for different mammal genera.

Table 2.3. Percentage base frequencies across all nucleotide sites, and variation in base frequencies at 1st, 2nd, and 3rd codon positions including codon position bias. Calculated using Equation 2.1 (page 44) from Irwin *et al.* (1991).

Codon position	**1**				**2**			
Base	**A**	**C**	**G**	**T**	**A**	**C**	**G**	**T**
Mean(%)	33.56	26.98	18.98	20.49	21.23	28.93	11.35	38.49
St. Dev.	0.2	0.38	0.17	0.35	0.16	0.29	0.1	0.27
Bias	0.14				0.23			

Codon position	**3**				**ALL SITES**			
Base	**A**	**C**	**G**	**T**	**A**	**C**	**G**	**T**
Mean(%)	34.32	43.44	6.68	15.56	29.81	33.02	12.35	24.83
St. Dev.	0.8	0.88	0.82	0.84	0.99	0.02	0.45	1.44
Bias	0.37							

The null hypothesis of neutral evolution of the data was not rejected by the McDonald and Kreitman test (G=0.256 P=0.612). Rate constancy could not be rejected by the relative rate tests (P>0.05) within the ingroup, or between the ingroup and the outgroups, suggesting consistency in the rate of evolution across taxa. A Shimodaira-Hasegawa test found no significant difference (P=0.6409) between the likelihood scores of the unconstrained tree (lnL = -5021.1273) and a tree in which a molecular clock model was enforced (lnL = - 5026.6035). Thus, the null hypothesis of no variation in overall rate constancy could not be rejected. Similar levels of sequence divergence in different clades can also be interpreted as evidence of similar age for the purposes of molecular clock calibration (e.g., Wüster *et al.*, in press). Tree length distribution, determined from random sampling of 10^6 unweighted trees was significantly skewed to the left (g1 = -1.179; P<0.01; Hillis, 1991; Hillis and



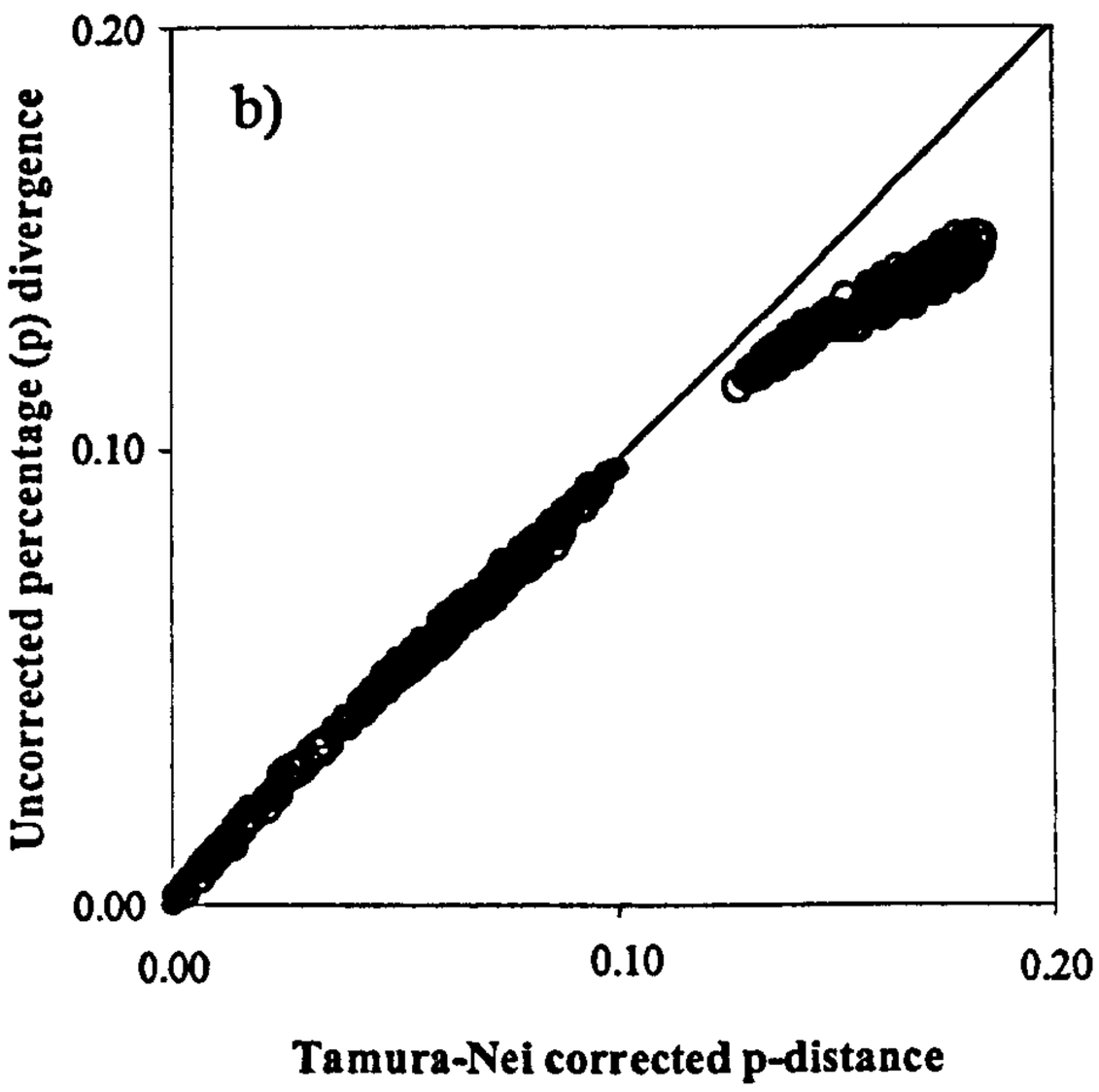


Figure 2.5. Qualitative assessment of the level of saturation of transitions at a) the 1st and 2nd codon positions and b) the 3rd codon position of mitochondrial cytochrome *b* and ND4 sequences in *Crotalus viridis*.

Huelsenbeck, 1992), suggesting strong phylogenetic signal in the data. Saturation plots (figures 2.5.a and 2.5.b) show no significant deviation from the isometric line at any positions, except for a minor deviation with 3rd base transitions, suggesting slight but

negligible saturation for these positions. Plotting uncorrected divergences against a more sophisticated evolutionary models (e.g. the general time reversible with rate heterogeneity) did not make any significant difference to the 3rd position transition deviation.

2.3.3 Phylogenetic reconstruction

2.3.3.i *Parsimony*

The heuristic analysis produced 35,146 most parsimonious trees (length 546; CI=0.6502; HI=0.3498; RI=0.8884), the bootstrapped consensus of which is shown in figure 2.6. Bootstrap values are shown above the branches, and Bremer support values below. Estimates of interior branch lengths were calculated using the interior- branch test (Rzhetsky and Nei, 1992; Nei *et al.*, 1998) in PHYLTEST (Kumar, 1996), and a high CP value of 1 indicated that all interior branch lengths were significantly greater than zero, thus supporting the accuracy of the estimated phylogeny (Sitnikova *et al.*, 1995).

2.3.3.ii *Maximum likelihood*

The best evolutionary model predicted by Modeltest (Posada and Crandall, 1998) was the TrN + proportion of invariable sites + gamma (Tamura and Nei, 1993; Tamura, 1994), a submodel of the general-time-reversible model (e.g., Yang *et al.*, 1994), with rate heterogeneity, which gave a likelihood score of lnL = - 4822.68, and recommended the parameters shown in Table 2.4. An improved score (InL = - 4805.4030) was achieved in the first likelihood analysis (tree 1) while enforcing the Modeltest parameters, and yet further improvement achieved with the new parameters estimated from tree 1 (Table 2.4). In each analysis base frequencies were A = 0.30; C = 0.33; G = 0.12; T = 0.25, and the proportion of invariant sites = 56 %. The bootstrapped phylogeny is shown in figure 2.7.

Table 2.4. Summary of maximum likelihood results

Likelihood score	**Rate matrix** ([A-C]) ([A-G]) ([A-T]) ([C-G]) ([C-T]) ([G-T])	**Gamma shape parameter**
Tree 1 lnL = -4805.4030	Modeltest parameters 1.00, 20.58, 1.00, 1.00, 12.84, 1.00	0.797
Tree 2 (best tree) lnL = -4804.0351	Parameters estimated from tree 1 0.56; 13.75; 0.75; 0.61; 8.14; 1.00	0.847

The consistency of the results from each analysis supports a single phylogenetic hypothesis for *Crotalus viridis*, in particular when the basal nodes of the phylogeny are considered (figs. 2.6 and 2.7). Two basal clades are apparent within the *C. viridis* complex. The first includes the populations of *C. viridis viridis* and *C. v. nuntius*, with a distribution east and south of the Rocky Mountains, and the second includes all remaining taxa west of the Rocky Mountains. The second most basal separation is the split of *C. v. cerberus* (Arizona), forming a sister lineage to the remaining western taxa. Bootstrap and Bremer support are high for both these basal nodes. There is generally low support for the internal structure of the *C. viridis-C. v. nuntius* clade, highlighting the close relationship of *C. v. viridis* and *C. v. nuntius* haplotypes within the clade. The remainder of the western group consists of two main divisions, one clade including haplotypes from the Great Basin region (*lutosus-abyssus*) and northern Pacific (*oreganus*), and a second clade consisting of two sister groups from the southern Pacific and Colorado Plateau (*caliginis-helleri* and *concolor*) respectively. Branch support for the arrangement of the major western partitions (excluding *C. v. cerberus*) is low. This might explain why in the present phylogeny the position of the Great Basin and northern Pacific groups are transposed compared to the first

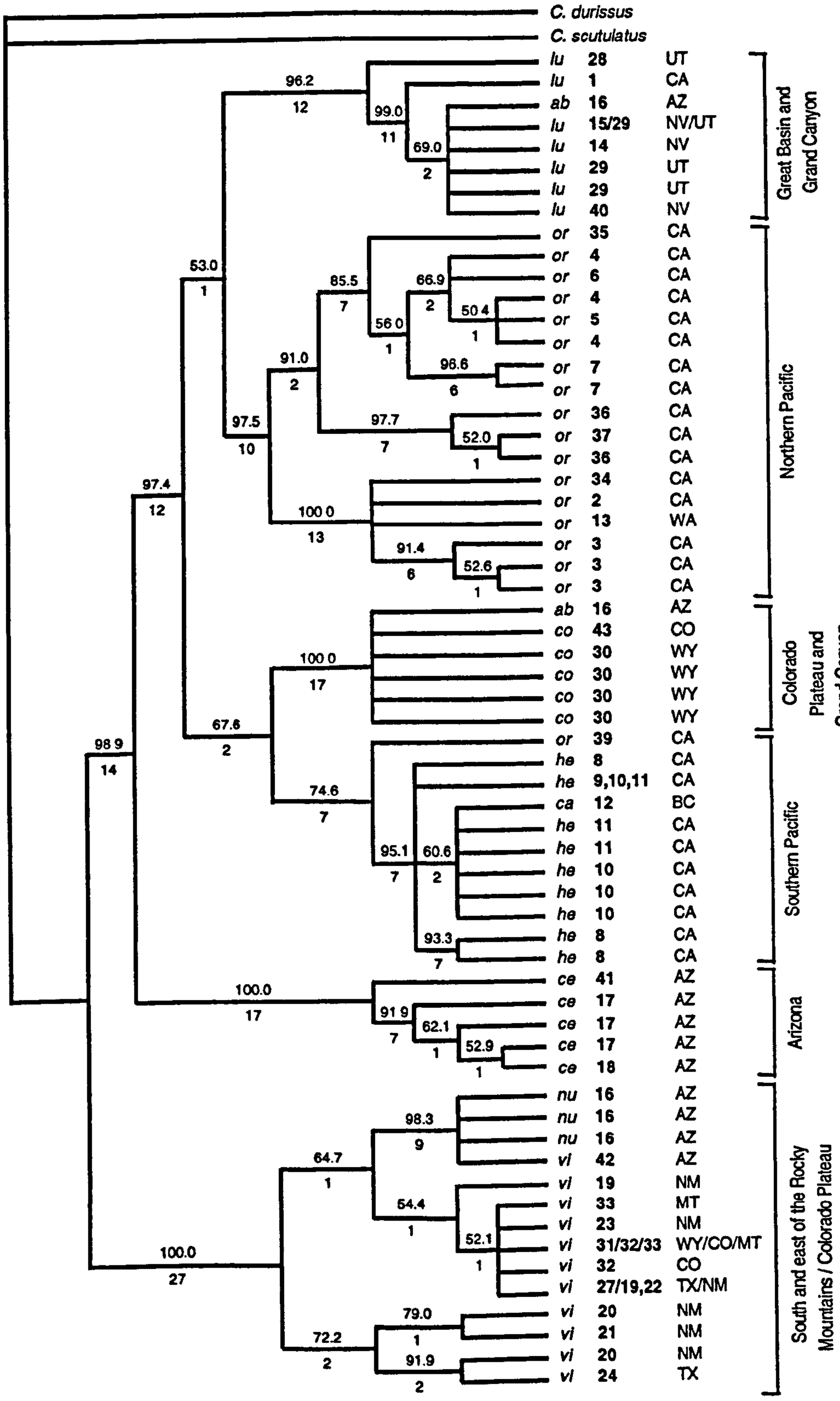


Figure 2.6. Strict consensus maximum parsimony phylogeny for the mitochondrial cytochrome *b* and ND4 genes in *Crotalus viridis*. Numbers above the branches represent bootstrap support and numbers below represent Bremer support. Terminal labels: *italics* = subspecies; **bold** = locality ref (see figure 2.2); upper case letters = US or Mexican state.

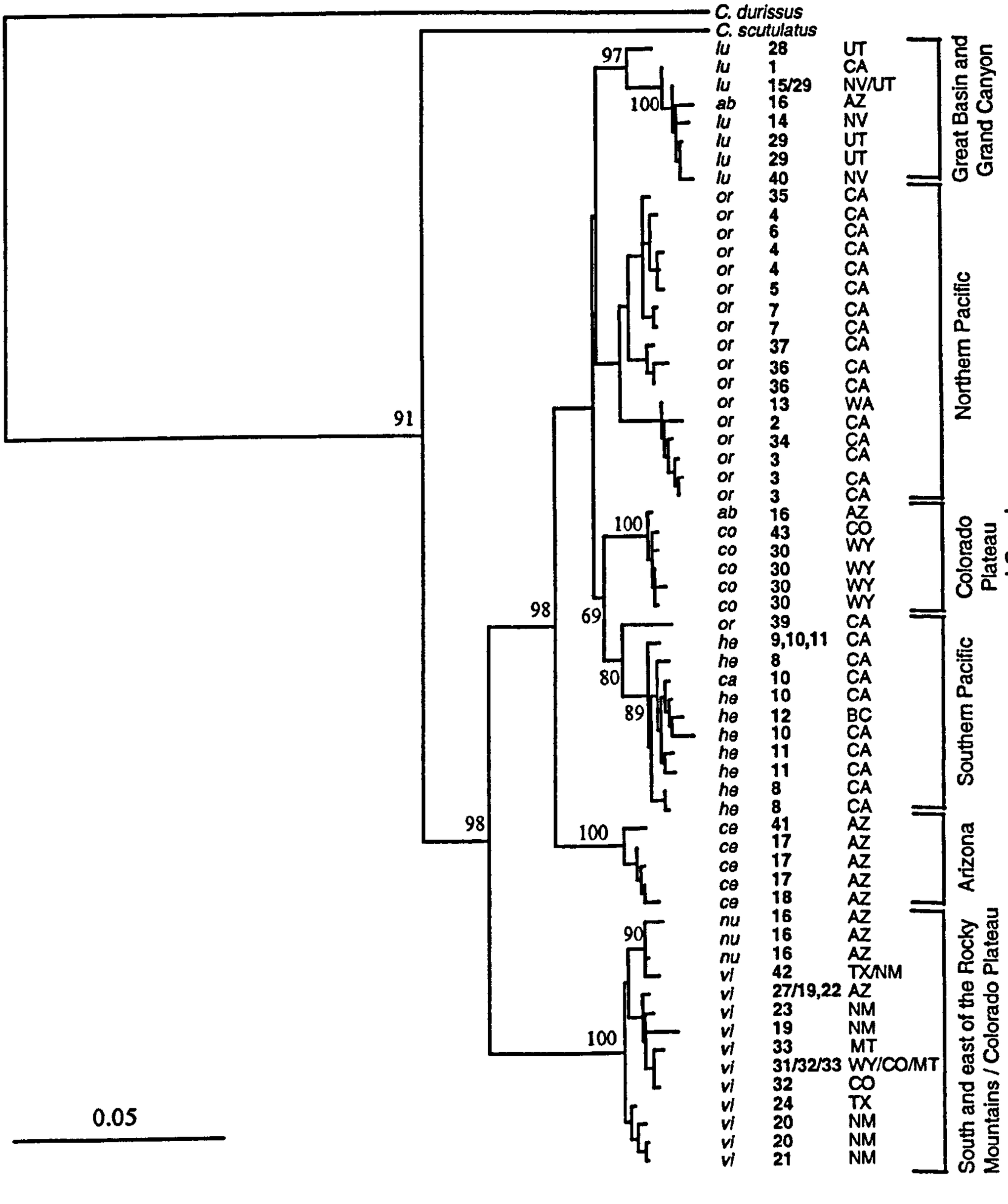


Figure 2.7. Maximum likelihood phylogeny for the cytochrome *b* and ND4 genes in *Crotalus viridis,* lnL= -4804.0351. Numbers at the nodes represent bootstrap support from 100 replicates. In the consensus bootstrapped phylogeny the relationship among the northern and southern Pacific, and Great haplotypes was unresolved. Terminal labels: *italics* = subspecies; **bold** = locality; upper case letters = US state.

phylogeny of Pook *et al.* (2000). Branch support for the grouping of haplotypes from similar localities is generally high, as might be expected, but resolution is lost towards the tips of each respective clade due to the very low levels of variation between the member OTUs of each clade. *C. v. cerberus* appears to form a long, separate lineage, and support for the position of *C. v. cerberus* in the phylogeny is high. The only unexpected phylogeographic pattern is the grouping of *C. v. concolor* (a Colorado Plateau population) within the Pacific-Great Basin group, although this clade is not strongly supported by the bootstrap (67.7%).

2.3.4 Tests of alternative phylogenetic hypotheses

The results of the Wilcoxon signed-ranks are summarised in Table 2.5. All results were found to be statistically significant, suggesting that in each case the data rejects all the alternative phylogenetic hypotheses of subspecies monophyly, plesiomorphy of Concolor toxin, small body size, and common causes of phylogenesis in favour of the most parsimonious interpretation. Similarly highly significant results were achieved from the Shimodaira-Hasegawa tests (Table 2.6) comparing the alternative likelihood hypotheses, suggesting that the difference in likelihood score is not due to chance, and thus also rejecting all hypotheses.

Table 2.5. Results of the Wilcoxon signed-ranks tests for three alternative phylogenetic hypotheses. A significant result indicates that the data rejects each alternative hypothesis in favour of the most parsimonious trees. TL1 is unconstrained most parsimonious tree length; TL2 is constrained most parsimonious tree length. Refer to Appendix III for trees.

Hypothesis	TL1	TL2	N	Z	*P*
i) Subspecies monophyly	440	452	40 – 45	-4.027 – -4.236	<0.0001
ii) Ancestry of Concolor toxin	440	455	27 – 33	-2.873 – -3.272	<0.01
iii) Single evolution of small size	440	505	61 – 65	-6.548 – -6.971	<0.0001
iv) *Pituophis* phylogeography imposed on *Crotalus*	449	550	45 – 47	-7.998 – -8.147	<0.0001
v) *Phrynosoma* phylogeography imposed on *Crotalus*	447	657	88 – 96	-5.835 – -5.978	<0.0001
vi) *Crotalus* phylogeography imposed on *Pituophis*	392	428	36 – 41	-4.959 – -4.697	<0.0001
vii) *Phrynosoma* phylogeography imposed on *Pituophis*	370	394	22 – 26	-3.870 – -4.070	<0.0001

Table 2.6. The results from the tests of alternative maximum likelihood hypotheses using the Shimodaira-Hasegawa test.

Tree	-ln L	d (–ln L)	*P*
Crotalus tree unconstrained	4804.0351		
i) Subspecies monophyly	4906.5940	102.5588	<0.0001
ii) Ancestry of Concolor toxin	4827.5287	23.4935	0.0197
iii) Single evolution of small size	4948.9044	144.8693	<0.0001
iv) *Pituophis* phylogeography imposed on *Crotalus*	7897.2889	3093.2538	<0.0001
v) *Phrynosoma* phylogeography imposed on *Crotalus*	5960.9341	1156.8890	<0.0001
Pituophis tree unconstrained	2853.8883		
vi) *Crotalus* phylogeography imposed on *Pituophis*	2993.9921	140.1078	<0.0001
vii) *Phrynosoma* phylogeography imposed on *Pituophis*	3042.9116	188.1117	<0.0001

2.3.5 Molecular clock estimates

Calibrating a new clock for *Crotalus viridis* does improve upon the new squamate calibrations proposed by Wüster *et al.* (in press). The Rocky Mountains provide the only reasonable (but highly speculative) vicariant explanation for the most basal separation in the *C. viridis* phylogeny (Pook *et al.*, 2000). According to Hunt (1974), uplift of the Rocky Mountains started in the early Cenozoic Era (65 million years ago), but the main altitudinal structuring of the mountains took place later, towards the end of the Tertiary period (Miocene or Pliocene). A specific estimate cannot be made, therefore, since the exact time period over which the Rocky Mountains became a potential barrier to dispersal is not known, and probably spanned several million years. Unfortunately the fossil record is also too sparse to be of any value (Holman, 1995).

Molecular clock estimates based on the *Porthidium* clock of Wüster *et al.* (in press) date the *C. viridis* lineage back as far as the late Miocene-early Pliocene (Table 2.7). It would seem that differentiation into eastern and western forms, closely followed by separation of *C. v. cerberus* from the remaining western taxa, probably took place early in the history of the lineage, late Miocene to early Pliocene.

Table 2.7. Time table of cladogenesis in *Crotalus viridis* using *Lachesis* and *Porthidium* mitochondrial cytochrome *b* and ND4 clock calibrations. Abbreviations: *vi* = *Crotalus viridis viridis*; *ce* = *C. v. cerberus*; *scu* = *C. scutulatus*; Mio = Miocene; Plio = Pliocene.

		Lachesis **(% My^{-1})**			***Porthidium*** **(% My^{-1})**	
	p-dist	0.60	1.06		1.09	1.77	
E-W	5.54	9.24	5.23	Late Mio early Plio	5.09	3.13	Early to mid Plio
ce-W	3.07	5.11	2.89	Early to mid Plio	2.81	1.73	Mid to late Plio
vi vs *scu*	7.67	12.78	7.23	Mid to late Mio	7.04	4.34	Late Miocene

2.4 Discussion

2.4.1 Mitochondrial DNA phylogeny and systematics of the *C. viridis* complex

The results reveal a well-resolved phylogeny for *Crotalus viridis*, and identify a number of haplotype clades, which, judging from their levels of sequence divergence, represent long-separated lineages. As is often the case in studies of this kind, the distribution of the mitochondrial haplotypes (figures 2.6-2.8) is not fully congruent with the conventional subspecies, in this case those recognised by Klauber (1972). Whereas the populations of some subspecies form distinct clades, others are rooted deep within clades of other subspecies. Thus, *C. v. helleri* and *C. v. viridis* haplotypes are paraphyletic with respect to *C. v. caliginis* and *C. v. nuntius* respectively, and *C. v. abyssus* haplotypes are polyphyletic, with one haplotype rooted within the *C. v. lutosus* clade and a second haplotype rooted within the *C. v. concolor* clade. Thus, out of the nine conventional subspecies, six are either paraphyletic, or insignificant local variants rooted deep within other clades. Highly significant Wilcoxon signed-ranks test and Shimodaira-Hasegawa test results further rejects the hypothesis of monophyly

of the subspecies haplotypes in favour of the topology in which subspecies monophyly is violated.

Recognition of the nine conventional subspecies of *C. viridis* as categories of equal rank, therefore, masks and confounds the strong phylogenetic pattern present. Clearly, the subspecies cannot be regarded as categories of equal rank, since some appear to be local varieties without independent phylogenetic histories, whereas others appear to constitute separate lineages with a long history of independent evolution. The clear separation of populations east and west of the Rocky Mountains, and the presence of *C. v. cerberus* as an isolated population in Arizona, suggests that these populations may represent separate evolutionary species.

2.4.2 Evolution of small body size

It can be deduced from the phylogeny that small adult body size evolved twice independently in *C. viridis*: once in the eastern clade (*C. v. nuntius*) and once in the Pacific Coast clade (*C. v. concolor*), a hypothesis which is strongly supported by highly significant Wilcoxon signed-ranks and Shimodaira-Hasegawa tests. This convergence offers excellent opportunities for the testing of the causes, correlates and consequences of the evolution of small body size in snakes.

2.4.3 Evolution of venom composition

The populations of *C. v. concolor*, with highly lethal venoms containing Concolor toxin (in adults), are rooted within the Pacific Coast and Great Basin Clade. The ability to secrete this particular component appears to have arisen *de novo* within this part of the complex. This hypothesis is strongly supported by statistically significant Wilcoxon signed-ranks tests, which show that the data supports the most parsimonious tree more strongly than the alternative phylogenetic hypothesis of plesiomorphy of

Concolor toxin. It is interesting to note that the other dwarfed population of the complex, *C. v. nuntius*, is not known to secrete Mojave toxin-like venom components.

This is the first analysis in which the phylogenetic position of rattlesnake populations secreting Mojave toxin-like phospholipases A_2 relative to non-secreting populations is assessed. Variation in this characteristic is known in other rattlesnake species (e.g., Glenn and Straight, 1978, 1985b, 1990; Gutiérrez *et al.*, 1991) and similar analyses in these other taxa may help to elucidate the causes and correlates of the ability to secrete this highly toxic venom component. This issue is also of the utmost importance with respect to improving the treatment of snakebite.

Although the rooting of *C. v. concolor* with the Pacific coast rattlesnakes is difficult to comprehend from a geographical point of view, it is congruent with previous results on venom composition. Despite the presence of Concolor toxin and its associated properties in *C. v. concolor*, Foote and MacMahon (1977) noted greater similarity in the venom profiles of *C. v. concolor* and southern Pacific rattlesnakes (*C. v. helleri*) than between *C. v. concolor* and those from the Great Basin (*C. v. lutosus*). This result is consistent with the phylogenetic position of these taxa as presented here. However, the results are inconsistent with the pattern of venom variation (figure 5.3, Chaper 5) in which *C. v. concolor* venom types form a separate cluster from those of the Pacific Coast. Furthermore, Young *et al.* (1980) note that the venom profiles of *C. v. abyssus*, *C. v. nuntius*, *C. v. lutosus*, and *C. v. concolor* showed only small differences, which are consistent with the minor morphological differences noted by Klauber (1972). Another interesting observation from venom studies is the closer similarity of *C. v. cerberus* venom to *C. v. viridis* than to *C. v. lutosus* (Foote and MacMahon, 1977), despite the fact that *C. v. cerberus* is the sister taxon to the remaining western populations. It would therefore be of interest to compare venom profiles from other western populations to that of *C. v. cerberus*.

2.4.4 Biogeography

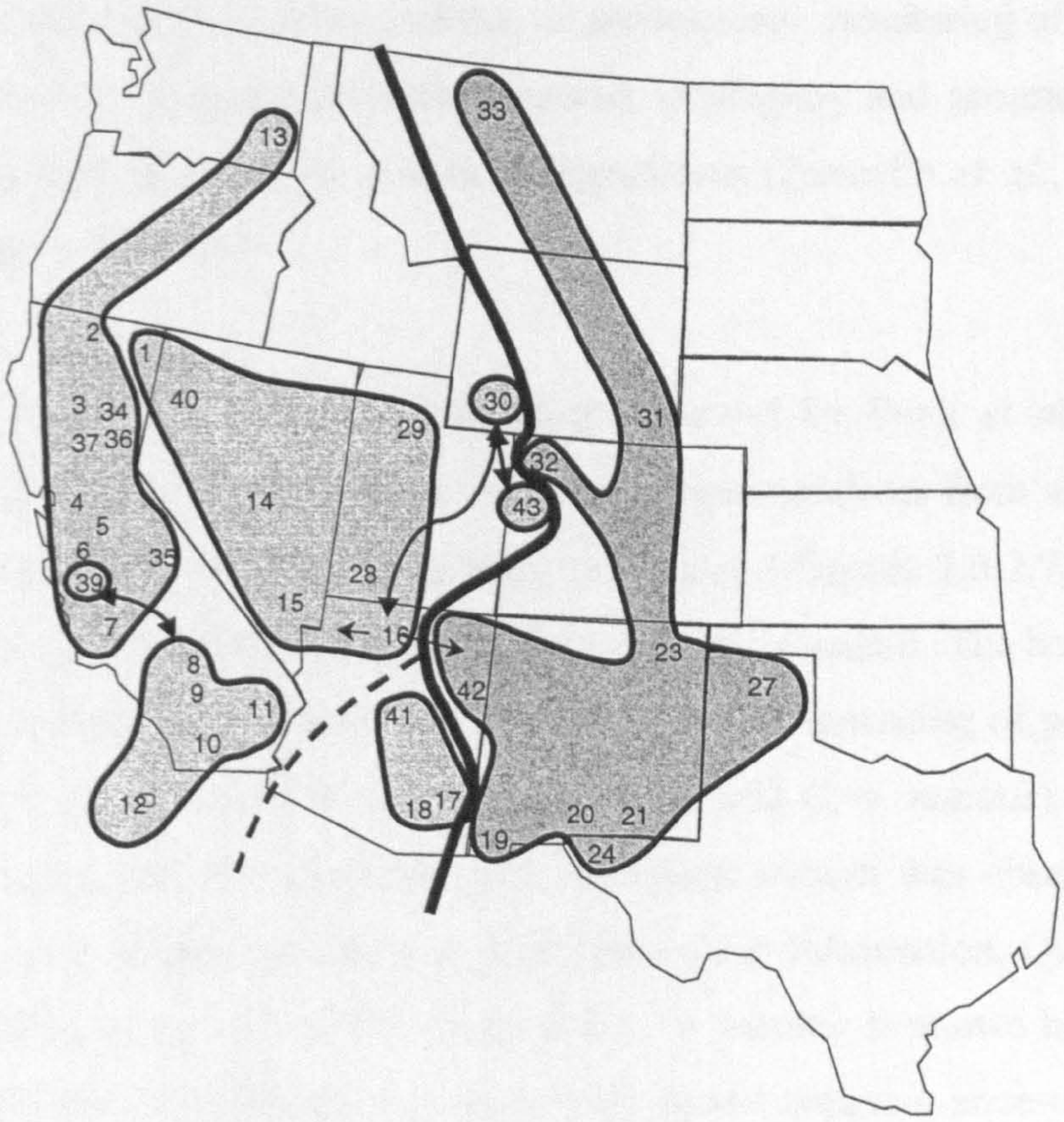


Figure 2.8. Distribution of mitochondrial haplotypes of cytochrome *b* and ND4 in *Crotalus viridis* in the western USA and Mexico. Numbers correspond to the locality numbers detailed in figure 2.2. See fig. 1.10 for state labels Locality 16 (Coconino County) is represented by haplotypes from three populations, conventionally *C. v. abyssus*, *C. v. lutosus*, and *C. v. nuntius*). Double ended arrows indicate that haplotypes from localities 30 and 43 form a cladewith one haplotype from the Grand Canyon, and 39 appears in the southern Pacific, rather than northern Pacific clade (see main text for details). The thick black line indicates the basal separation of the eastern and western clades (see phylogenies in figs. 2.6 and 2.7). The dotted line demarcates separation of *C. v. cerberus* haplotypes from the other western clades.

Many other studies of North American herpetofauna (e.g., Lamb *et al.*, 1989; Zamudio *et al.*, 1997; Barber, 1999; Orange *et al.*, 1999; Shaffer and McKnight, 1996; Wiens *et al.*, 1999) have revealed marked patterns of phylogenetic structuring of populations in the western USA, strong concordance between phylogeny and geography, and often can be explained in terms of vicariance hypotheses (Zamudio *et al.*, 1997; Barber, 1999; Orange *et al.*, 1999).

The distinct pattern of regional structuring observed by Pook *et al.* (2000), in a phylogeny of 19 ingroup haplotypes (including representatives from all 9 subspecies populations), is maintained in the present phylogeny (ffigures 2.6-2.7), in which the sample size of most subspecies populations is greatly increased. The basal split within the species complex separates *C. viridis* into a lineage consisting of populations east and south of the Rocky Mountains (*C. v. viridis* and *C. v. nuntius*) and a western lineage, including all the remaining taxa. The same pattern was observed by Quinn (1987) based on limited molecular and morphological information. Quinn also noted the close association of *C. viridis viridis* and *C. v. nuntius* as shown here, supporting the idea that these populations may share very recent common ancestry. The second separation is a split between *C. v. cerberus*, a central Arizona form, from the rest of the western populations.

The main clades show strong concordance with the major geographic areas of the western United States (figs. 2.6-2.8). According to the molecular clock estimates, the main period of divergence of *Crotalus viridis* occurred between 9.24 and 1.73 Ma (Table 2.7), in the mid-Miocene to late-Pliocene. Geological events throughout this time, including uplift of the North American western Cordillera, associated with aridification of the west, explain most effectively vicariance within the *C. viridis* complex. In particular, Arizona up to and including the Four Corners area of the Colorado Plateau (where the states of Arizona, Utah, Colorado, and New Mexico meet), appears to represent an important centre of phylogeographic partitioning within this species, since at least six of the conventional subspecies occur in this region (*C. v. abyssus*, *C. v. cerberus*, *C. v. concolor*, *C. v. nuntius*, *C. v. lutosus* and *C. v. viridis*).

From the estimated times of divergence (Table 2.7), it would seem that the eastern lineages may have separated from the western lineages during the late Miocene to mid Pliocene (3-9 Ma.). The tectonic activity responsible for the uplifting of the Rocky Mountains had virtually ended; however, there continued an extended period of significant volcanism in the Southwest associated with the formation of the plateaux now associated with the Grand Canyon, and in particular the Colorado Plateau (Hunt, 1974; Whitney, 1996). Extensive lava flows and deposition of volcanic ash (throughout the late Tertiary and Quaternary periods) have played a significant role in sculpturing the Colorado Plateau landscape, even into more recent times in the Pleistocene (Hunt, 1974).

The geological activity associated with the Colorado Plateau and surrounding area, together with the shift in vegetational distribution, appears to coincide with the estimated time of the basal split between *C. viridis* east and south of the Rocky Mountains (*C. v. viridis* and *C. v. nuntius*) and the remainder of the complex. The most southwesterly extremity of the current range of the *C. v. viridis*-*C. v. nuntius* clade corresponds to the mountainous zone of the Canyon and Datil Section of the Colorado Plateau, which roughly defines the Plateau's southern rim. In the past, the volcanic activity around the Grand Canyon may have presented a barrier to gene exchange between eastern (Rocky Mountain-Colorado Plateau) and western (Great Basin-Pacific Coast) clades. Extreme volcanic activity is believed to have taken place around the southern edge of the Colorado Plateau, and around the Grand Canyon, throughout the mid-Miocene to mid-Pliocene. Opportunities for gene flow would also have been fewer at that time, due to the reduced number of tributary canyons connecting the Grand Canyon with other regions (Hunt, 1974).

The low level divergence between the northern and New Mexico *C. v. viridis* suggests only recent divergence within the clade. The present day distribution of northern populations is most likely the result of postglacial range expansion; during the Pleistocene glacial stages a considerable part of the Midwest was affected by the continental ice-sheet, which covered northern Montana, and the eastern half of North

and South Dakota in the Wisconsinan glacial stage, and which reached as far as the northeastern corner of Kansas in the Nebraskan and Kansan glacial stages. There is also evidence of glaciation in the higher parts of Arizona (Hunt, 1974), and unsuitable climatic conditions presumably extended considerably further south.

During the same time period (3-6 Ma.), a significant climatic change occurred resulting from the Sierra Nevadan - Cascadian orogeny, which initiated the process of aridification in the west (Potts and Behrensmeyer, 1992; Whitney, 1996; Brown, 1997). Increasing aridity, together with the rain-shadow effect of the Rocky Mountains (Axelrod, 1985; Potts and Behrensmeyer, 1992) caused a gradual replacement of largely semi-open sclerophyllous woodlands to shrubsteppe, open and desert grasslands (Axelrod, 1985; Keeley and Keeley, 1989; Sims, 1989; Brown and Wright, 1975; Zamudio *et al.*, 1997), which, east of the Rocky Mountains, marked the start of the expansion of the Great Plains (Axelrod, 1985). Further differentiation of the east and southeast Rocky Mountain populations most likely, therefore, involved adaptation in concert with the gradually expanding grassland and desert shrub habitat associated with climatic change.

The present distribution of *C. v. cerberus* lies within the southern limits of the Basin and Range Province, and is delimited by the southern rim of the Colorado Plateau, and the Central Highlands, Arizona. This distribution is consistent with the sister-group relationship with the western as opposed to the eastern forms found in our phylogeny. The separation and isolation of the *C. v. cerberus* lineage from the remaining western populations also falls within the late Miocene to early Pliocene, but appears to have followed between 2 to 4 Ma. after the divergence of the eastern lineage. Continued volcanic disturbance spreading into the Basin and Range Province (Hunt, 1974), together with a rapid expansion of regional deserts associated with the aridification process (Riddle, 1995), restricted the forests to cooler, moister, upland areas (Potts and Behrensmeyer, 1992) with large expanses of inhospitable terrain in between (Whitney, 1996). Consequently, in the late Miocene to mid Pliocene, populations of *C. viridis* became isolated in relic montane forests in Arizona, prevented from redispersal. These

isolated populations gave rise to modern *C. v. cerberus*, which is found only in woodland habitats. At present there is no reliable evidence of contact with other members of the species group.

Interpretation of the remaining western clades corresponding to the Pacific and Great Basin regions is more difficult. The present results would suggest more recent divergence of these lineages, and it is possible that gene flow continued throughout the Pacific and Great Basin regions during the formation of the Sierra Nevada and Cascade Mountains. These mountain chains now appear to form substantial barriers between the two regions, although an intergrade zone is known to exist in Idaho, lying between *C. v. oreganus* in Washington state and *C. v. lutosus* in Nevada (see Chapter 3). Gene exchange could continue from Washington, south through Oregon and into California. Limited gene flow may also be possible through a pass in the Sierra Nevada Mountains in northeast California. Similarly an intergrade zone exists between *C. v. oreganus* and *C. v. helleri* where the ranges of these populations meet (Klauber, 1972; see Chapter 3). The colonisation of South Coronado Island (off the northwest coast of Mexico), traditionally recognised as *C. v. caliginis*, is clearly a recent event, and the only haplotype from this island is contained within the clade of the southern coastal forms (*C. v. helleri*). It is likely that the island was occupied during the repeated cycles of eustatic lowering of sea levels coincident with Pleistocene glaciations. The status of *C. v. caliginis* as a subspecies must be regarded as open to question (see General Discussion).

The sister-group relationship between the widely separated southern Pacific coast populations (*C. v. helleri*), and those from southwestern Wyoming and extreme western Colorado (*C. v. concolor*), appears geographically incongruent. However, Bremer support for this relationship is weak. If substantiated by further studies, it may be explicable as vicariance due to the continued aridification of the southwestern USA during the latter part of the Pliocene to early Pleistocene (Hunt, 1974). This hypothesis would suggest the original existence of a continuous population extending from southern California up to Colorado River Valley. The final stages of aridification and habitat change (Webb, 1977) around the mid to late-Pliocene (2-3 Ma.) would have

led to the extinctions of populations in the lower and middle Colorado River Valley, and the existence of relict populations on the Colorado Plateau and the mesic parts of southern California. The middle course of the Colorado River, especially the Grand Canyon, was later occupied by populations originating from the Great Basin (*C. v. lutosus*, *C. v. abyssus*), which may now be contiguous with *C. v. concolor*. A phylogeographic pattern uniting populations from southern California and the upper Colorado River might also be found in other wide-ranging, non-hot-desert species of similar age in the southwestern USA.

It is ill advised to rely solely on mitochondrial haplotypes to define species, or subspecies boundaries, since even apparently strong geographical differentiation may not be reproducible with a different marker (Barber, 1999; Thorpe and Richard, 2001). The importance of considering alternative evidence, such as nuclear markers and/or morphology before definitive changes are recommended, cannot be overemphasized (e.g. Thorpe, 1996; Cannatella *et al.*, 1998). Sites and Crandall, 1997 stress the importance of avoiding *ad hoc* interpretations of phylogeny, and highlight the need for comprehensive empirical hypothesis testing under rigorous, predefined criteria when considering species concepts (for reviews see Cracraft, 1989; Templeton, 1989; Frost and Hillis, 1990; de Queiroz, 1998). Ultimately, however, consideration will need to be given to the species status of some populations presently included in the *Crotalus viridis* complex, since species recognition is of importance with respect to biodiversity inventory (Sites and Crandall, 1997), species conservation (e.g. McElroy, 1995; Avise, 1998; Bernatchez and Wilson, 1998), and, in the case of venomous snake species, toxinological research and the development of efficient antivenoms for the treatment of bites (Wüster and Thorpe, 1991, 1992; Wüster and McCarthy, 1996; Wüster *et al.*, 1999a).

Chapter 3. Patterns of Variation in Morphology

3.1 Introduction

3.1.1 Geographic variation

Geographic variation, the occurrence of differences among spatially segregated populations within species, is found in nearly all groups of organisms. The degree and patterns of geographic variation vary considerably for different species, and in some instances variation may be highly localised to among neighbouring demes, for which the term microgeographic variation has been coined (Mayr, 1970; Gould and Johnston, 1972; Thorpe, 1987; Malhotra and Thorpe, 1991a). The existence of geographic variation has been acknowledged since the Linnaean period or earlier (Mayr, 1970; Endler, 1977). Classical evolutionary and taxonomic thinking, however, was highly "typological" (Mayr, 1970). Taxonomists believed in arranging organisms into unvarying, discrete categories such as species, then subspecies. The acceptance of geographic variation to replace "typological thinking" was therefore pivotal in the development of the modern view of evolution (Futuyma, 1986). In turn many questions have been raised regarding species concepts and how the "species" unit should be defined (see de Queiroz, 1998), especially with the emergence of new perspectives arising from molecular analyses. The interpretation of patterns and potential causes of geographic variation has therefore become an integral part of contemporary intraspecific systematic studies.

3.1.2 Patterns of geographic variation

Many characters are known to be subject to geographic variation (Mayr, 1970; Thorpe, 1987). The most observable variation is morphological, but variation may also be detected in all other possible character systems, including biochemical or molecular character systems, even in apparently morphologically indistinct populations

(Futuyma, 1986; Thorpe, 1987). Given the comparatively recent advent of biochemical and molecular techniques, morphological character systems have been by far the most important in investigations of species relationships (Miyamoto and Cracraft, 1991), forming the basis for the definition of specific structural patterns of geographic variation. The main types of patterns include clines, categorical variation, transition and hybrid zones. A cline is a gradual, unidirectional change in the character with space. Clines are referred to as stepped, when a sharp step occurs within an otherwise gradual cline (Grant, 1972), or when two uniform areas separated by a transition zone (Endler, 1977). Categorical variation (disjunction) may refer to any variation in which distinct categories can be identified, and encompasses categories of island populations, and allopatry or parapatry in mainland populations. Transition or hybrid zones entail a rapid transition between relatively stable geographic races (Endler, 1977; Thorpe, 1987). These main pattern-types apply to both Rassenkreis (polytypic) and Artenkreis species (non-interbreeding parapatric species or superspecies) (Endler, 1977; Futuyma, 1986). Other types of pattern include mosaic and ecotypic variation amongst others, which are described in Endler (1977) and Thorpe (1987). Since patterns of geographic variation are not always categorical, acceptance of arbitrary subspecies categories may mask the real population affinities. If the pattern of geographic variation is a smooth cline there is little point in arbitrarily sectioning a species into subspecies (Thorpe, 1980c, 1984) but if there are distinct racial categories then the use of trinomials can possibly be justified (Thorpe, 1985c).

The pattern of geographic variation can be confused by other, non-geographic forms of intraspecific variation, including sexual dimorphism, ontogenetic (developmental), or temporal variation (Thorpe, 1976). For example, head dimensions in some snakes, e.g. *Nerodia sipedon* (water snakes), *Vipera berus* (adder), and *Crotalus viridis* are sexually dimorphic, but also appear to be associated with adaptation to available prey size in different areas (Klauber, 1972; Benton, 1980; King, 1989; Forsman, 1994; Queral-Regil, 1998). Character systems often involve complex functional interactions (Thorpe, 1989; Thorpe and Brown, 1989; Malhotra and Thorpe, 1991a; Thorpe *et al.*, 1994). Colour patterns in *Anolis* lizards, for instance, appear to be functionally

partitioned, in that one set of characters is concerned with sexual selection, others impart crypsis (Malhotra and Thorpe, 1991a), while others are associated with species recognition (Leal and Rodríguez-Robles, 1995). In *Crotalus viridis* growth rate varies ontogenetically, but also this species exhibits sexual dimorphism, and geographic variation with respect to altitude and latitude (Macartney *et al.*, 1990). The effects of sexual dimorphism may be counteracted by treating males and females separately, and to account for the effects of allometry each group should include a representative spectrum of sizes (Thorpe, 1976; Willig *et al.*, 1986).

3.1.3 Multivariate methods

Multivariate techniques such as geographic networks, contouring and isometric plots (Thorpe, 1976), colour mapping, and transects (Thorpe, 1984), are based on the spatial distribution of the population localities. Other methods are not based on spatial distribution, such as cluster analysis, and ordination techniques such as factor analysis, principal component, principal coordinate, single discriminant, and canonical variate analysis (Thorpe, 1975b, 1976, 1987). In many of these methods, the degree of similarity between each pair of OTUs is determined from a matrix of similarity coefficients. A popular choice of coefficient is the Mahalanobis (1936) generalised square distance (D^2), favourable because to a certain extent, it accounts for intra-locality (within-group) correlation between characters, thereby overcoming some of the problems of information redundancy (Thorpe, 1976; Benton, 1980).

3.1.3.i *Ordination methods*

Ordination methods may be applied to all major categories of geographic variation in the generalised phenotype, which is expressed in terms of vectors (eigenvectors). Vectors may either be interpreted directly to describe morphological variation within species, geographic origin, race, or ecological niche of the OTUs, or alternatively, a

visual interpretation may be made of the racial affinities of discrete groups of OTUs, formed when the vectors (usually the first two or three) are plotted against each other on a scatter diagram. The first approach considers the vectors independently, whilst the second and preferred approach, considers the vectors in relation to each other (Thorpe, 1979, 1980a).

3.1.3.i.a Principal components analysis (PCA)

Principal components analysis is used to ordinate character states of individual specimens (rather than groups) or the mean character-states for a particular locality (Benton, 1980). PCA is generally carried out on covariance or correlation matrices from which the principal components are extracted. The first principal component defines an axis along the greatest dimension of the scatter, and thus expresses the greatest amount of variation. Subsequent principal components, orthogonal to the other principal components, express progressively less variation (Thorpe, 1976). Any characters measured in different units must be standardised to zero means and unit standard deviation prior to PCA if being used for taxonomic purposes. Standardisation serves to prevent variation in some characters from being "weighted" more than others, for example when some values depart widely from the mean. PCA does not take into account within-locality correlation between characters, except in the case where the principal coordinates are extracted from a suitable D^2 similarity coefficients matrix, whereupon the analysis, by definition, is a canonical analysis. PCA is a less powerful means of discrimination than canonical variate analysis, but has the advantage of making fewer assumptions about the data than CVA. The data does not require *a priori* grouping, and heteroscedastic data (data with unequal variance/covariances) may be used (Thorpe, 1976, 1979; Neff and Smith, 1979; Fowler and Cohen, 1993; Sokal and Rohlf, 1999).

3.1.3.i.b Canonical variate analysis (CVA; discriminant function analysis)

The more rigorous of the ordination methods, canonical analysis ordinates *a priori* groups of individuals. The maximum variation between groups is sought, thus maximising group separation whilst minimising the overlap between groups. A group may include specimens from a single locality, compound locality, or many localities, although a group comprising a local population is preferable for the analysis of geographic variation.

An advantage of canonical analysis is that, because the method is based on Mahalanobis D^2 similarity coefficients, within-group covariation between characters is taken into account, thus eliminating redundant information in a character set. Furthermore, CVA takes into account both between group and within group variation to maximise group separation. Unlike PCA, however, CVA requires homoscedastic data (the groups are assumed to have equal variance/covariances). It is important to ensure that groups do not contain multiple species, therefore this must be tested beforehand. CVA is the preferred method over PCA because it can simultaneously ordinate all of the groups' members as well as the group means. However, a prerequisite of this preference is good congruence between a PCA of group means and the mean CVA scores for the same groups using the same character system. If the results of the PCA and CVA conflict, because of heteroscedasticity, it is advisable to refer only to the more robust PCA (Thorpe, 1976). CVA also requires large sample sizes, and is therefore this method is difficult to apply to rare or poorly represented taxa.

For reviews and examples of all the methods described above, see Thorpe (1976, 1980a, 1987); Neff and Smith (1979); Manly (1991).

3.1.3.ii *Character selection*

Characters should be selected which are significantly informative with respect to geographic variation. Another important consideration is the level of congruence between patterns of geographic variation of different characters between localities (i.e. between-locality correlation). Higher levels of congruence between resulting patterns for different character sets, allow more robust causal hypotheses of variation to be formulated, than if causal hypotheses were based on a few characters alone. Given that congruence is influenced by genetic or ontogenetic dependence however, the chosen characters must not only be genetically or ontogenetically independent, but also show low intra-locality correlation (or low pooled within-sample correlation), since highly intra-locality correlated characters inevitably lead to congruent patterns of geographic variation and thereby information redundancy (Thorpe, 1976, 1984, 1987).

Whilst only a few significant characters are required to achieve a particular geographic pattern (as demonstrated by Thorpe, 1985d with respect to clinal variation), the presence of non significant characters will decrease congruence between the character sets compared, and the number of significant characters examined has to be increased several fold. Although the decrease in congruence with the addition of non significant characters is slight, these characters are best excluded from the study (Thorpe, 1985d).

3.1.3.iii *Sample sizes and pooling localities*

Prior to multivariate analysis consideration should be given to the sample sizes for each geographical location (e.g., Thorpe, 1979, 1984; Wüster and Thorpe, 1992). Most importantly the sample for a particular locality should constitute a fully representative complement of within-locality variation. Hence the appropriate sample size for different localities may vary according to the level of within-locality variation. In cases where sample size is very low for a particular locality, and the level of intra-

locality variation is not known, localities should be pooled. Pooling localities should not be carried out across species or subspecies, otherwise character weighting may be biased in CVAs (Thorpe, 1980b). There is a common tendency for subjective and arbitrary group construction using predefined categories such as subspecies or colour (e.g., Bauer and Branch, 1995; Reichling, 1995; Schneider et al., 2001), but such a practice erroneously assumes within-group homogeneity, that the defined group consists of one species or subspecies (Wüster and Thorpe, 1992). The presence of two species is not always self-evident from a superficial examination of specimens (e.g., Thorpe and McCarthy, 1978). Use of these *a priori*-defined groups may, therefore, mask potentially important characters. Given the disadvantages of low sample size, however, pooling represents a compromise between being forced to exclude a sample altogether, thereby reducing the representative within-group variation further and not knowing whether that sample would made a significant contribution to the separation of populations, or including that sample in the hope that it will make an appropriate contribution to the separation of populations if allocated correctly.

3.1.4 Morphology of *Crotalus viridis*

The intraspecific relationships of *C. viridis* have been difficult to describe in terms of morphology. Brattstrom (1964) did not find osteological characters especially informative, and vaguely suggested that *C. v. concolor* and *C. v. lutosus* seem closely related, likewise *C. v. helleri* to *C. v. oreganus*. In many other characters, however, *C. v. oreganus* more closely resembled *C. v. viridis* than either *C. v. lutosus* or *C. v. helleri*. Neither does the fossil record help to elucidate the relationships within *Crotalus viridis*, due a great paucity of fossil evidence, being represented solely by vertebrae from the late-Pleistocene to recent from just a handful of localities in California, Nevada, Nebraska, Kansas, and Colorado, and one locality in Iowa. One suspected Lower Pliocene vertebra is recorded from Nebraska (Brattstrom, 1954; Holman, 1995).

Presently, geographic variation in *Crotalus viridis* is described by nine subspecies (figures 1.1-1.10, Chapter 1), which are differentiated by colour pattern, body size, and three lepidotic (scale) characters. These characters, however, are not entirely consistent. The latest revision of the subspecies was summarised by Klauber (1972), who himself acknowledges the subjective nature of subspecies classification, and that the definition between populations becomes progressively lost as the ranges of populations start to merge.

In rattlesnakes the background dorsal colour generally conforms to the substrate on which the animal lives, and the dorsal blotch patterns have a cryptic, rather than aposematic function (Klauber, 1972). Colour pattern can vary, however, not only geographically, but ontogenetically, and possibly with temperature (Klauber, 1930, 1949, 1956, 1972). *Crotalus. v. cerberus*, for example shows an ontogenetic change from an elaborate and contrasting pattern in the juvenile, to almost complete melanism in the adult, although the degree of melanism may vary with locality. The colour pattern of some populations of *C. v. helleri* also darkens towards adulthood, although in these populations melanism is thought to be associated with wetter environments and/or higher altitude. The distal broad band on the tail of juvenile *C. v. helleri* is a characteristic yellow, which darkens to brown or black within the first year. In contrast *C. v. abyssus* and *C. v. concolor* lose definition in the dorsal pattern with age, fading to a more uniform coloration. Other ontogenetic changes include the fusing and melanism of the head markings of *C. v. helleri*, and the "bleaching" of the markings on the lateral part of the head below the eye in the Coast Range populations of *C. v. oreganus*, resulting in a characteristic white face (Klauber, 1949). Broad variation in colour pattern has also been observed at the intra-locality level, particularly in *C. viridis*, including *C. v. viridis, C. v. nuntius, C. v. cerberus,* and *C. v. oreganus.* Consequently Klauber (1972) suggests that colour is not a reliable character for distinguishing variation. To complicate matters further, *C. v. viridis*, *C. v. helleri*, and *C. v. cerberus* are also capable of temporary colour change due to the effect of temperature on the distribution of melanin (black pigment) in the melanophores (black pigment cells). Higher temperatures causes melanin to become concentrated towards

the centre of the melanophores, leading to paling of colour, and to an extent increased contrast in pattern. At cooler temperatures and the melanin disperses within the pigment cells, and the overall colour pattern darkens (Pough *et al.*, 2001).

Klauber (1972) observed a tendency towards higher scale counts and pale background coloration in arid compared to lower scale counts and increased melanism in wetter environments. Scale counts are suggested to increase with body size, with an additive effect where scale counts become higher still when large body size is combined with living in an arid area. Klauber also suggests that selection for crypsis appears to dominate over effects of climate in situations where these conditions conflict, for example rattlesnakes inhabiting arid areas but living against dark substrates tend to be dark in colour rather than pale. However, Klauber also points out that the colour pattern trends associated with climate generally hold when species are compared, but become inconsistent when populations within species are compared. Although these hypotheses are interesting, none have been rigorously tested statistically.

These observations raise two main questions regarding selection pressures on scalation or colour pattern, which clearly require further investigation. First, the question arises whether scalation is a developmental trait influenced by temperature. Klauber (1972), for example, refers to the study of Wade Fox (1948) in which pregnant garter snakes from the same locality, but artificially maintained at different temperatures, produced babies with higher scale counts at warmer than at cooler temperatures. This is not the only example where temperature is found to influence embryonic development in reptiles (e.g. body size - Voss, 1993; sex - Bull *et al.*, 1982; Wibbels *et al.*, 1991; Wibbels *et al.*, 1998; behaviour - Berger, 1989; Shine *et al.*, 1997a; locomotor performance - Shine *et al.*, 1997a).

3.1.5 Aim

This chapter aims to test for the existence of discrete morphological groupings within *Crotalus viridis*, and to describe patterns of geographic variation in morphology, using quantitative, multivariate analysis of scalation, dimensional, and pattern characters. The resulting patterns of morphological variation are contrasted with the conventional subspecies categories presently used to describe geographic variation in *Crotalus viridis*, and the nature of the transition between the subspecies is examined. The observed patterns are subsequently used as a foundation for formulating and testing causal hypotheses of intraspecific variation in the next chapter.

3.2 Materials and methods

3.2.1 Specimen acquisition

Preserved specimens (n = 522) of *Crotalus viridis* from across the species range were examined from North American museums and university collections. The sample included males (n=313) and females (n=209) of a range of ages. Voucher specimens were taken from the field where appropriate, and preserved or maintained in captivity. Details of all specimens examined are given in Appendix IV. Morphological data were recorded, where possible, for animals from which tissue and venom samples had also been taken. In many instances, however, recording morphological data for these individuals was not permitted, due to welfare regulations regarding anaesthetising animals, or if the same animals were being used in other projects. To compensate for being unable to record morphology of live snakes, data were taken (as far as possible) from museum specimens from the same localities.

3.2.2 Selection of Characters

In total, 60 characters were selected (33 meristic and 27 linear - see below) with low within-sample correlation, representing scalation (n=28), pattern (n=16), body dimension (n=7) and "miscellaneous" (n=9) character systems. The characters chosen were:

1 shown in previous morphological studies to differentiate between populations of *C. viridis* (Klauber, 1956, 1972; Quinn, 1986)

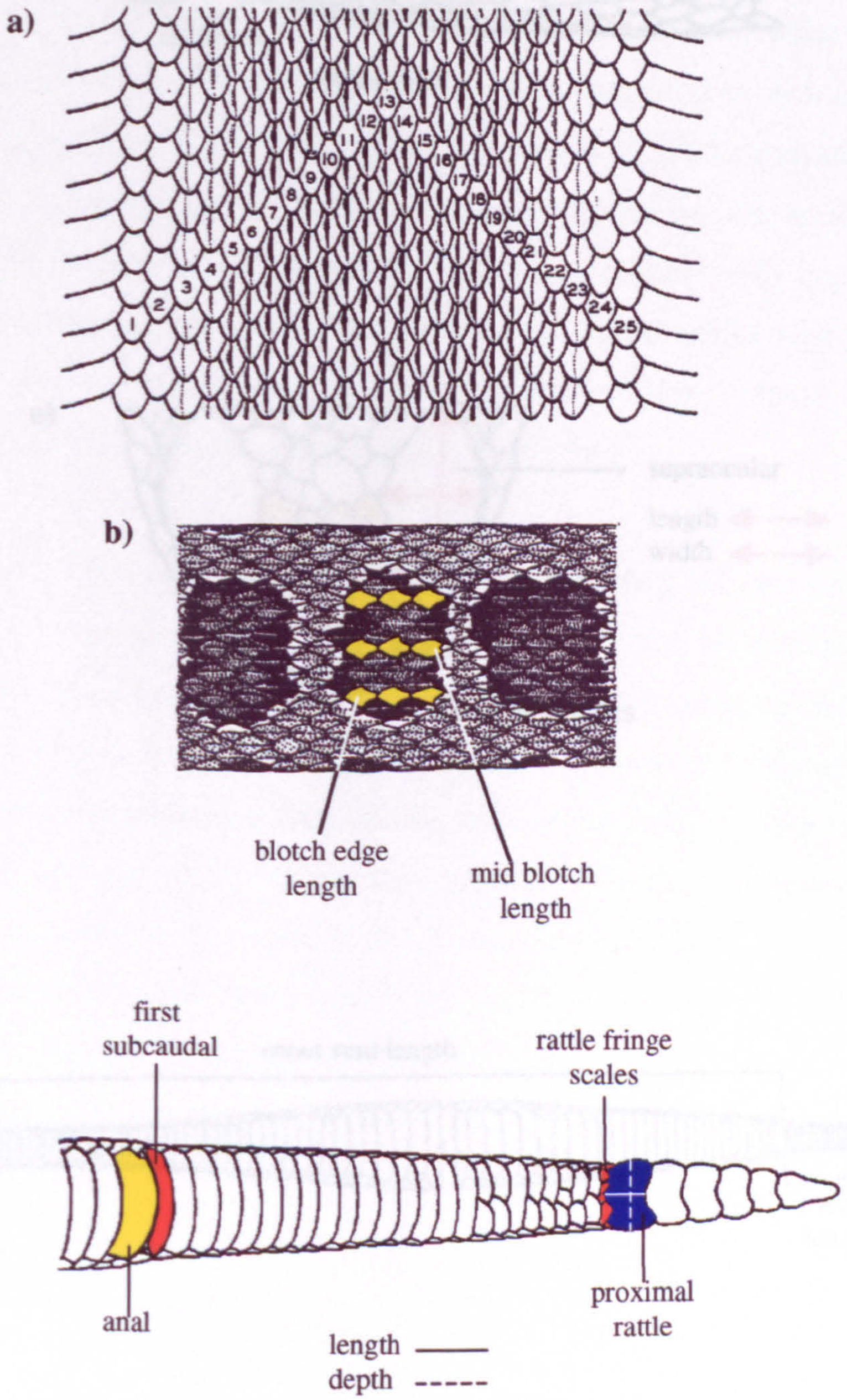
2 shown to be useful in elucidating the intraspecific relationships of other rattlesnake taxa, e.g. *C. lepidus* and *C. triseriatus* (Dorcas, 1992) and *C. willardi* (Barker, 1992)

3 shown to be significantly geographically variable in a preliminary sample of preserved specimens of *C. viridis* representing different populations

4 considered important with respect to ecological niche, such as pit size, eye size, mouth width etc.

Snout-vent-length was measured with the aid of a piece of string laid along the mid-ventral line, from the tip of the snout to the posterior edge of the anal scale. All other linear measurements were recorded with a pair of Mitutoyo digimatic callipers (Mitutoyo U.K. Ltd.). Where appropriate, characters were measured in scale counts to avoid allometric effects. The position of a character along the body was scored as the number of the ventral scale opposite which it was situated. In order to compensate for different ventral scale counts, this was converted to % ventral scale count (% VS). Where relevant, all head scale counts and measurements were taken for both left and right sides and the average recorded. Blotch edge lengths, measurements and counts, were recorded one scale row in for both left and right edges (figure 3.1.a) for three blotches at 25, 50, and 75% VS along the length of the body, and the overall average of the six values calculated. Likewise blotch length (along the mid-line-dorsal scale row, figure 3.1.a), and blotch width (in number of dorsal scales) were recorded for three blotches at each position and averaged respectively. Blotch

Figure 3.1. Some morphological scale characters and dimensions recorded for *Crotalus viridis*, modified from Klauber (1972). **3.1.a.** illustrates how dorsal scale rows were counted, **3.1.b.** dorsal scale counts for blotch length, **3.1.c.** tail characters, **3.1.d.** and **3.1.e.** head scale characters, **3.1.f.** body characters/dimensions. Refer to text for additional details.



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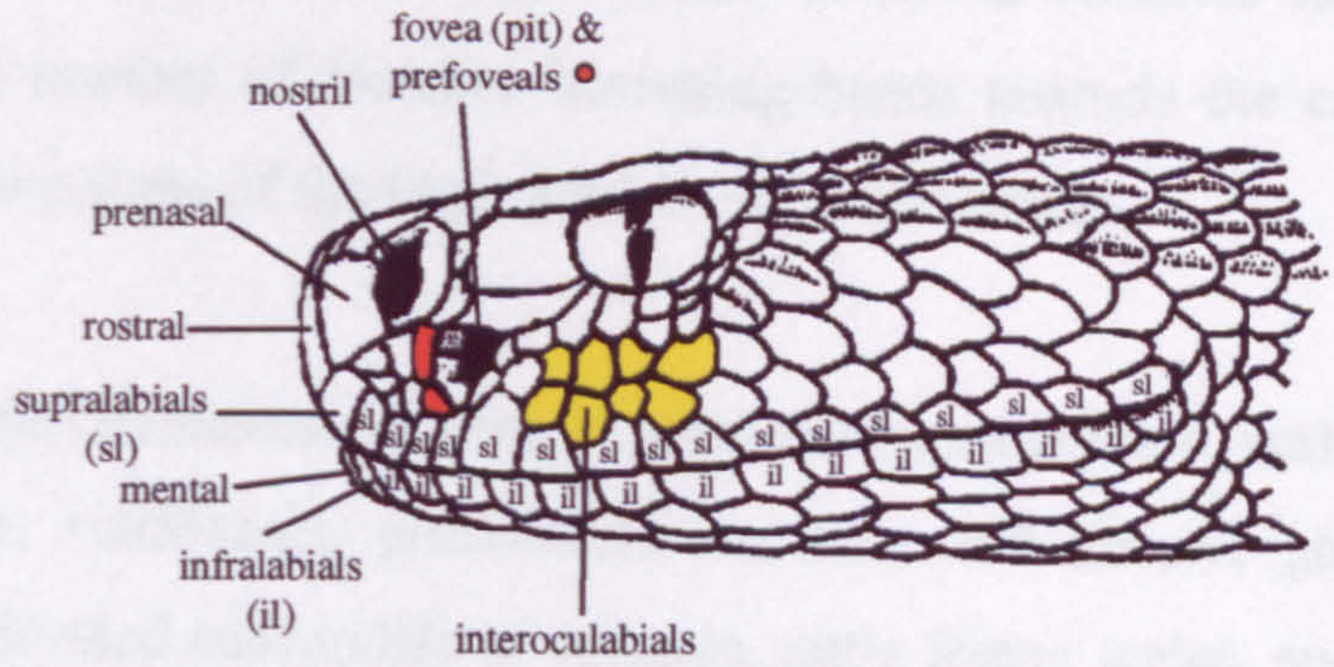
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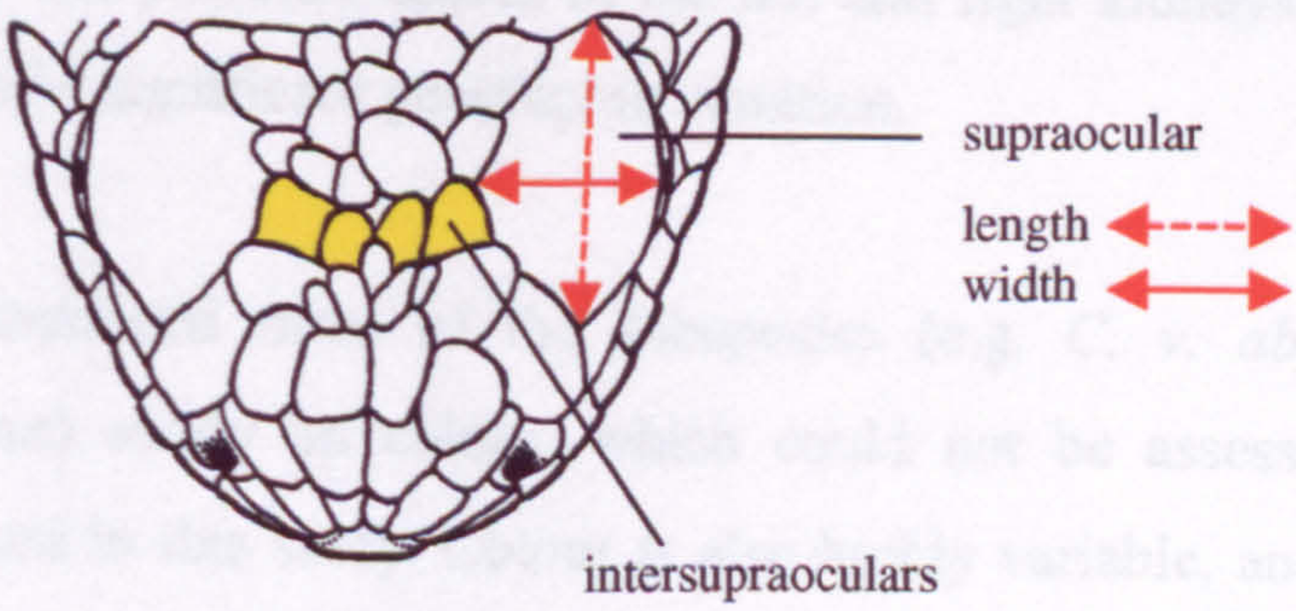
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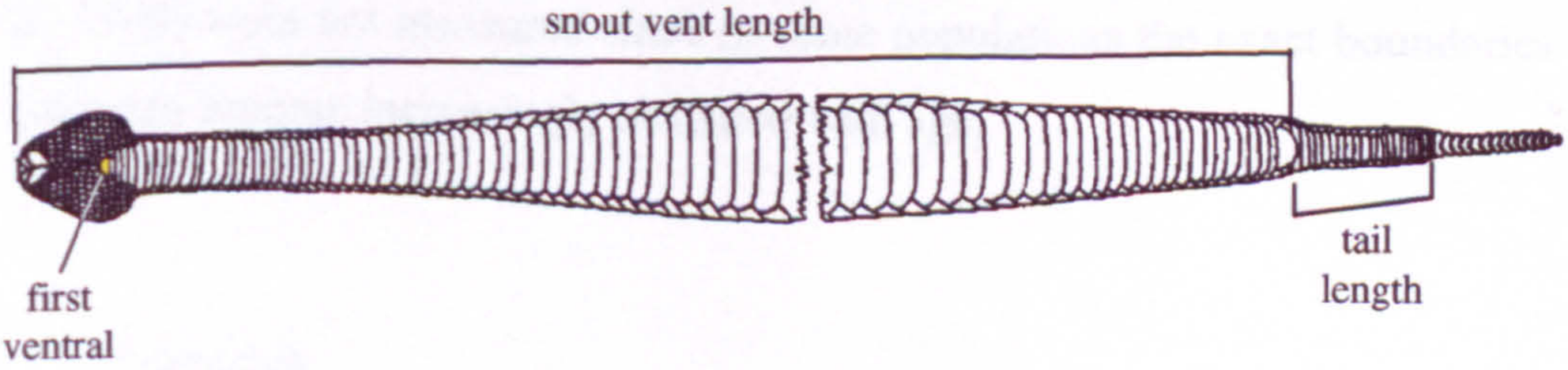

snout vent length
first
ventral
tail
length

lengths were standardised to percentage of ventral scales, and blotch widths to percentage of dorsal scale rows. The number of dorsal blotches fused with lateral blotches (i.e. the number of blotches becoming bands towards the caudal end) were presented as a percentage of the total number of body blotches.

Additional meristic characters, number of postoculars, suboculars, scales bordering the supraocular scale, subfoveals, postfoveals, canthals and loreals, gular scale rows, percentage of undivided subcaudals or ventrals, rattle fringe scales, and the number of scales between blotches at 25, 50 and 75% did not show significant variation between populations in a preliminary analysis of variance comparing ten localities. These characters were removed from subsequent analyses. Similarly body organ positions (measured in relation to the percentage number of ventral scales along the body), including the anterior and posterior apices of the left and right kidneys, heart, liver, and gonads, did not show significant geographic variation.

Klauber (1972) differentiated three of the subspecies (e.g. *C. v. abyssus*, *C. v. concolor*, *C. v. lutosus*) solely on colour, which could not be assessed from the museum specimens used in this study. Colour is also highly variable, and difficult to quantify, hence does not represent a sensible choice of character. In some populations of *Crotalus viridis* colour is also dramatically affected by ontogeny (e.g. melanism in adult *C. v. cerberus* and C. *v. helleri*, whitening faces in *C. v. oreganus*, Klauber, 1949) which is difficult to account for in multivariate studies (Thorpe, 1976). Consequently tail bands, and lateral face stripes (noted to vary between populations by Klauber, 1949) were not measured since in some populations the exact boundaries of these markings become increasingly obscured with age.

3.2.2.i *Lepidosis*

1-10 Number of dorsal scale rows at 10, 20, 30, 40, 50, 60, 70 80, 90, and 100% VS positions (figure 3.1.a).

11-13 Number of dorsal tail scale rows at 25, 50, and 75% CS positions (CS = caudal scale rows)

14 Number of ventral scales (using the procedure of Dowling, 1951) (fig.3.1.f)

15 Number of subcaudal scales (fig. 3.1.c)

16 Number of supralabial scales (fig. 3.1.d)

17 Number of infralabial scales (fig. 3.1.d)

18 Number of intersupraoculars (scales between the supraocular scales) (fig. 3.1.e)

19 Number of interoculabial scales (fig. 3.1.d)

20 Number of prefoveal scales (scales in front of the pit) (fig. 3.1.c)

3.2.2.ii *Individual scale dimensions*

21-22 Supraocular scale length and width (mm) (fig. 3.1.e)

23-24 Prenasal scale length and width (mm) (fig. 3.1.d)

25-26 Rostral scale length and width (mm) (fig. 3.1.d)

27-28 Mental scale length and width (mm) (fig. 3.1.d)

3.2.2.iii *General pattern*

29 Number of dorsal body blotches

30 Number of tail bands

31 Percentage of dorsal blotches fused with lateral blotches (becoming bands towards the tail)

32 Amount of ventral mottling: 1=none; 2=slight (1-33.34%); 3=medium (33.34-66.67%); 4=heavy (66.67-100%)

33-35 Length of edge of dorsal body blotches (dorsal scale row units) at 25, 50 and 75% VS positions (fig. 3.1.b)

36-38 Length of dorsal blotches at mid point (dorsal scale row units) at 25, 50 and 75% VS positions (fig. 3.1.b)

39-41 Blotch width (number of dorsal scale rows) at 25, 50 and 75% VS positions (fig. 3.1.b)

42-44 Length of dorsal blotches (mm) at 25, 50 and 75% VS positions

3.2.2.iv *Body dimensions*

45 Snout vent length (mm) (fig. 3.1.f)

46 Tail length (mm) (fig. 3.1.f)

47 Head length – from the articulation of the mandible with the quadrate (distal end of quadrate, proximal end of mandible) to the anterior edge of snout (mm)

48 Head width at widest point (gape) (mm)

49 Head width at eye level (mm)

50 Snout width immediately anterior to the eyes (mm)

51 Snout length - anterior margin of eye socket to anterior edge of snout (mm)

3.2.2.v *Other*

52 Pit diameter (mm) (fig. 3.1.d)

53 Eye diameter (mm)

54 Distance between eye and nostril (mm)

55 Distance between eye and pit (mm)

56 Distance between pit and nostril (mm)

57 Fang length

58 Proximal rattle length (anterior–posterior dimension) (fig 3.1.c)

59 Proximal rattle depth (dorsal–ventral dimension) (fig 3.1.c)

60 Proximal rattle width (left lateral–right lateral dimension)

3.2.3 Preliminary assessment of character data

All morphological analyses were carried out using the BMDP statistics package (Dixon, 1991). The subprograms used within BMDP are indicated in parentheses after the name of each statistical test.

3.2.3.i *Data error checks*

The data were checked thoroughly for errors. Erroneous meristic values were identified as those points which fell noticeably outside the main frequency distribution for each character. Scatter plots of points of the measured values against snout-vent-length were constructed to identify mensural outliers. The data summaries provided by BMDP (programs AM and 7M) were also useful for exposing unusual values representing potential sources of error, for example extremes in group means and standard deviations, extremes in Mahalanobis D^2 distances from the own-group centroid, or contradictory jackknife classifications.

3.2.3.ii *Construction of operational taxonomic units (OTUs)*

Given that sample sizes for many individual localities were low, and that some of these localities were scattered geographically, specimens from several localities had to be pooled into single operational taxonomic units (OTU). The following procedure was followed to avoid creating spatially heterogeneous groups (i.e. artificial groups with pronounced within-group geographic variation):

1 Males and females were assessed independently to avoid the influence of sexual dimorphism.

2 Meristic data alone were used to assign groups, since mensural data are complicated by the close linear relationship between character and size due to allometric growth, and require *a priori* grouping for proper analysis. Allometric effects of ontogenetic variation are negated by means of regression models, which integrate the morphological data obtained from all members of a regional group and inductively remove the effects of body size (Thorpe, 1976; Wüster and Thorpe, 1992), but this can only be achieved after a regional pooled OTU has been defined.

3 A broad range of meristic characters was used (n= 33: body scale counts, head scale counts, and colour pattern characters) to avoid biasing group selection towards a single character-type.

4 To establish which populations formed discrete homogeneous groups according to all the characters selected, the data were first standardised to zero mean and unit standard deviation, and subjected to principal components analysis (PCA). Groups resulting from an initial PCA of all samples (identified from two-dimensional plots of the first and second principal component scores of each specimen), were subjected to subsequent PCA to determine whether further separation could be achieved within those groups. The procedure was continued until no further significant separation could be achieved in any groups, at which point all the individuals forming a given group were declared to represent one compound locality. Consequently group (OTU) homogeneity was maximised by splitting any groups that appeared heterogeneous.

5 Individuals whose identity was uncertain, such as individuals from localities coinciding with potential intergrade zones (e.g. *C. v. oreganus* and *C. v. lutosus* in Idaho), were allocated to unique groups (per locality) irrespective of whether or not they appeared as discrete groups in the PCA. Group composition was also checked to ensure that the population members, even though deemed morphologically alike according to the PCA, were not in reality, separated by any obvious physiographical, geographical, or habitat barrier.

A total of 27 locality OTUs were defined (figure 3.2; Tables 3.1-3.2). As far as possible, the same group assemblages were applied to females and males, although some inconsistencies were encountered where certain localities were represented by only one sex. These localities, however, were not geographically remote from other localities represented by both sexes, and were checked for within-group homogeneity by PCA to ensure that there was no discontinuity with respect to group composition.

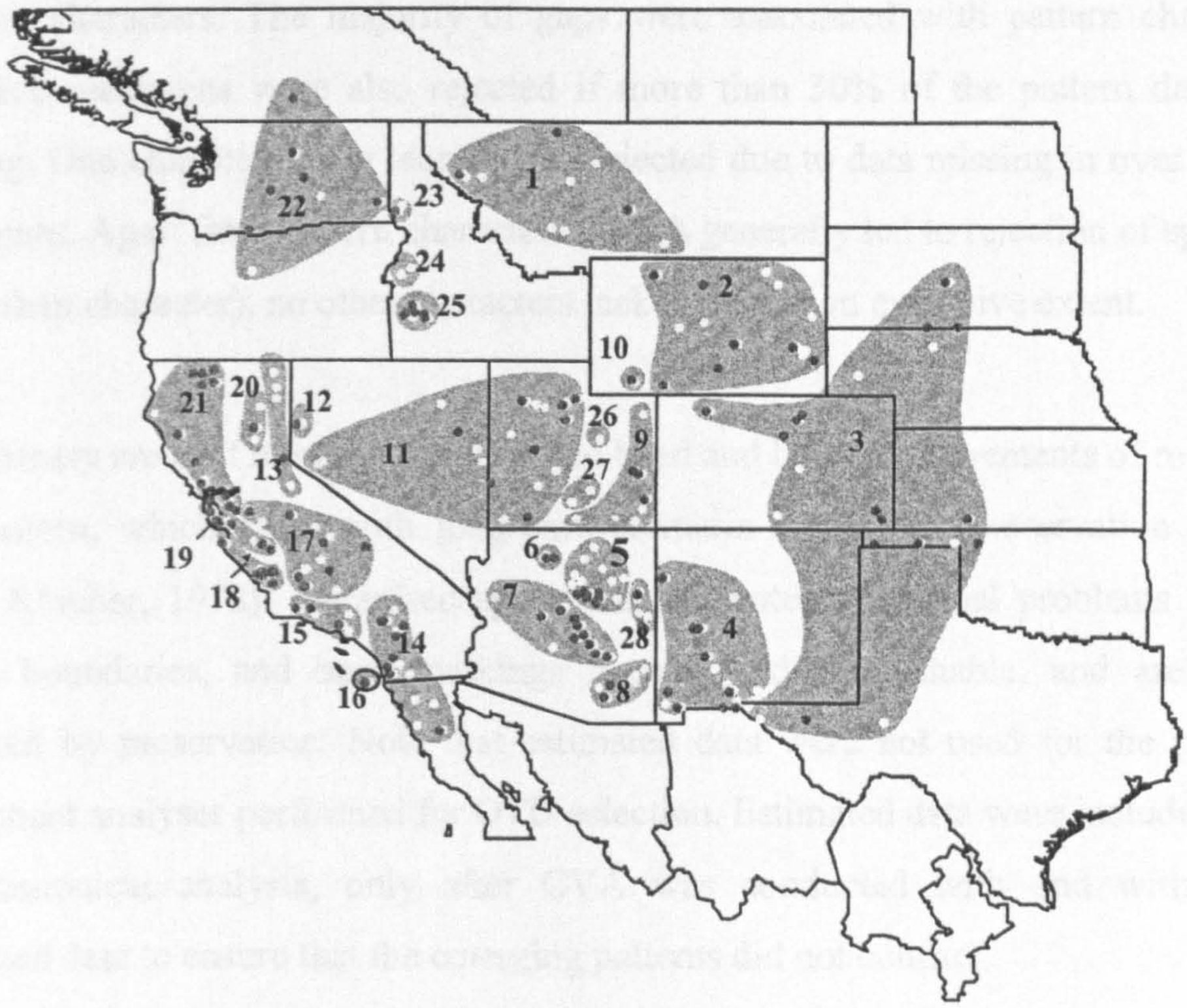


Figure 3.2. Locality pools for males (white points) and females (grey points), as determined by principal component analysis. Refer to Appendix IV, for museum accession numbers and locality details.

3.2.3.iii *Estimation of missing data*

Most analytical programs reject specimens with missing data, hence it is necessary to determine how many individuals are affected by a lot of missing data and whether group sample sizes are adversely affected as a consequence. Rather than reducing sample size, it may be preferable to reject characters with excessive missing data, although if the amount of data missing is small, the character may be estimated by regression analysis (BMDP AM program). In the present study, specimens were rejected for which missing data exceeded 20% of the total number of significantly

variable characters. The majority of gaps were associated with pattern characters, therefore specimens were also rejected if more than 30% of the pattern data were missing. One character (fang length) was rejected due to data missing in over 15% of specimens. Apart from pattern characters (which generally led to rejection of specimen rather than character), no other characters lacked data to an excessive extent.

The primary areas of missing data included head and body measurements of road kills, and pattern, which fades with long-term formalin or alcohol preservation (Gloyd, 1938; Klauber, 1972). Melanised specimens presented additional problems because blotch boundaries, and head markings become indistinguishable, and are further obscured by preservation. Note that estimated data were not used for the principal component analyses performed for OTU selection. Estimated data were included in the final canonical analysis, only after CVA was conducted with and without the estimated data to ensure that the emerging patterns did not conflict.

3.2.3.iv *Preliminary analysis of meristic data.*

Two-way analysis of variance (ANOVA; BMDP 2V; grouping criteria being sex and locality), was performed on males and females simultaneously to test which meristic morphological characters showed significant geographical variation between populations, and which characters exhibited sexual dimorphism. The ANOVA also reveals whether the interaction of locality and sex is important. In each case, characters failing to show significant between-group (locality) variation at the 5% level were rejected. To minimise reductions in sample size (due to rejection of individuals with missing data), subsets of characters were analysed independently. The results are summarised in Table 3.3.

3.2.3.v *Preliminary analysis of mensural data.*

Males and females were initially treated separately to avoid the influence of sexual dimorphism. Most characters are found to be highly, and positively correlated with snout-vent length (SVL), which consequently represents an appropriate measure against which to standardise body size. Each character was logarithmically transformed, giving a linear relationship with body size (Fowler and Cohen, 1993; Sokal and Rohlf, 1999). The regression model against body size varies for different characters (e.g., Rossman, 1980; Forsman, 1994; Queral-Regil, 1998), therefore alternative models were tested (BMDP 7M), log neither SVL nor character, log SVL only, log character only, log SVL and character, and the model giving the highest within-group correlation was considered to be the best model for that character. An initial assessment of sexually dimorphic and significantly geographically variable characters was made using two-way analysis of covariance (ANCOVA; BMDP 2V; Table 3.4) of all individuals, in which size was adjusted by specifying SVL as the covariate. The significance test of ANCOVA, therefore, involves a test for significant differences in size-adjusted means between populations (Sokal and Rohlf, 1995). Character selection was ultimately made, however, using one-way ANCOVA (BMDP 1V) for each sex independently. Characters were accepted for which the probability of adjusted means was statistically significant at the 5% level (i.e. characters show significant between-locality variation), and for which there was a non significant difference between the equality of slopes at the 5% level (i.e. for the regression *y* intercepts to be meaningful ANCOVA requires that the regression slopes are parallel). The final results are shown in Table 3.5.

3.2.4 Canonical variate analysis: generalised pattern and shape

Canonical variate analysis (BMDP 7M) was conducted initially on all selected mensural (size adjusted using the appropriate regression models) and meristic characters combined to determine the general pattern of geographical variation in

Crotalus viridis. The resulting discrete groups were then assessed independently to test whether any further separation could be achieved, although the procedure was halted once the results were reduced to a handful of groups with high morphological similarity and close geographical proximity. Further ordination at this stage almost inevitably results in the separation of the predefined groups, and therefore is no longer informative in discerning patterns of geographic variation.

3.2.5 Investigating the nature of contact zones

Present literature (e.g. Klauber, 1956, 1972) and unpublished results from two theses (Quinn, 1986; Schneider, 1986) suggest a zone of intergradation between *C. v. helleri* and *C. v. oreganus* in the central-southern part of California, and between *C. v. lutosus* and *C. v. oreganus* in Idaho. The exact nature of these proposed intergradation zones is not known. Two transects were therefore considered for each region, hereafter referred to as the 'California transect' and 'Idaho transect' respectively. A '*viridis-nuntius* transect' is also examined, based on the observations of Klauber (1935), and Quinn (1986) of a gradual north to south change in certain characteristics of the Prairie rattlesnake (*Crotalus viridis viridis*), particularly size, to Arizona, where the snakes are then referred to as *C. v. nuntius*. These populations are not only contiguous with each other, but also they are difficult to distinguish at the point where they meet on the western border of New Mexico and eastern border of Arizona (Klauber, 1935).

In each case sexes were treated separately, (although an Idaho transect could not be constructed for females because not all localities are represented). Transects were defined through the proposed clinal regions. Characters were selected using one-way ANOVA (for meristic characters) and one-way ANCOVA (for mensural characters), that showed significant variation between populations at each end of the respective transects, but excluding suspected intermediate forms. Refer to Tables V.1 and V.2, Appendix V for the character lists. Group structure was as follows:

3.2.5.i *California transect*

Defining a north-south transect from Washington state to Baja California, Mexico (figure 3.8.a), all Californian populations were included in the analysis (localities 14 to 22; males n=72; females n=58), which comprised individuals from Oregon (males n=1; females n=0), Washington (males n=8; females n=10), and Canada (males n=0; females n=4). Isla Coronado Sur (Los Coronados, Mexico) was also included (males n=9; females n=10).

Characters were selected based on all groups except populations from the southern part of the Central Valley (figure 3.8.a) of California (Kern, Fresno, Merced, Madera, Tulare, and El Dorado Counties), where the snakes display different characteristics from either the extreme northern or southern populations (Schneider, 1986), and areas known to have intermediate forms of *C. v. oreganus* and *C. v. helleri* (San Luis Obispo and Monterey Counties). The latter two populations are therefore considered to represent potentially intermediate forms.

Patterns of geographic variation were visualised using principal component analysis, and clinal variation assessed by plotting the first principal component against latitude. The resulting transect scatter plot was then related to a map of the area (figure 3.8).

3.2.5.ii *Idaho transect*

A transect was chosen from the north of Washington state, following the Snake River which runs exactly along the western border of Idaho where it adjoins the eastern boundaries of the states of Washington and Oregon. The southern edge of Idaho abuts the northern boundaries of the states of Nevada and Utah, however the Snake River takes a course to the east just before the southern state border, at the northern edge of the Great Basin region (figure 3.9.c).

The analysis included representatives from the Great Basin (Nevada, northeastern corner of California, Utah; n=24; conventionally *C. v. lutosus*), northern California west of the Sierra Nevada Mountain range (n=8; conventionally *C. v. oreganus*), Washington (n=7; conventionally *C. v. oreganus*), Ada and Elmore Counties, Idaho (part of the Great Basin region and south of the Snake River; n=10; conventionally *C. v. lutosus*), Adams and Washington Counties, Idaho (north of the Great Basin edge, and north of the Snake River, n=11; identity unclear), and Nez Perce and Latah Counties, Idaho (north of Adams County, bordering Washington; n=5; identity unclear). The data were analysed as for the Californian transect.

3.2.5.iii viridis-nuntius *transect*

The transect was defined between Montana in the northern U.S., south to the northeastern corner of Arizona (figure 3.10.a). Characters were selected which varied significantly between Montana and Arizona. Principal component analysis was conducted on all populations along the transect, Montana (males n=10; females n=4), Wyoming (males n=14; females n=7), Colorado (males n=11; females n=13), New Mexico (males n=18; females n=14) and Arizona (males n=34; females n=14). The data were assessed as for the previous two transects.

3.3 Results

3.3.1 Operational taxonomic units

In total, 27 geographically discrete, morphologically homogeneous groups were defined from scatter plots of the first and second principal components using meristic characters (see also Results for character selection), to subdivide 313 individual males and 209 females respectively (figure 3.2). Certain OTUs (Table 3.1) were defined *a priori* irrespective of whether they were contiguous with other populations in the PCA,

based on the criteria of disjunction, uncertain identity, or populations with ambiguous or unclear relationships in the PCA. The positions of all individuals allocated to these OTUs were nevertheless checked by PCA to ensure that they formed groups according to each respective locality. All remaining groups were determined directly from the PCA scatter plots, given in Appendix VI, figures VIa.i-ii to h.i.-ii. Individuals consistently forming large single, geographically widespread homogeneous groups in the PCAs included *C. v. viridis* (from Montana south to New Mexico) and northern Pacific *C. v. oreganus*. Since it is advisable to compare as many localities as possible, these groups were subdivided further according to geographical proximity, whilst maintaining suitable sample sizes so as not to compromise representative variation.

Table 3.1. OTUs defined on grounds of distribution, or uncertain identity

Conventional subspecies	Locality (OTU)	Justification for group
abyssus	Grand Canyon, Arizona (6)	Disjunct or restricted distribution
cerberus	Arizona: Central Highlands region (7) and Pinaleño-Galiuro-Santa Catalina Mts (8).	
concolor	Sweetwater County, Wyoming (10)	
lutosus	Anahoe Island, Pyramid Lake, Nevada (12)	
caliginis	Isla Coronado Sur, Los Coronados, Mexico (16)	
oreganus-lutosus	Washington (22) and Idaho (23)	Intergrade zone
viridis-concolor	southeastern corner of Utah (9)	
helleri-oreganus	California: Santa Barbara (15), Central Valley (17), Monterey and San Luis Obispo (18)	
uncertain	Utah: Carbon (26) and Garfield (27) Counties	Identity uncertain or conflicts in PCA results
uncertain	Idaho: Adams (24) and Nez Perce (23) Counties	

Table 3.2. Operational taxonomic units and sample sizes, defined using principal component analysis of meristic characters. Also refer to the map in figure 3.2.

OTU	Taxon	Locality notes	*n* Males	*n* Females
1.	*viridis*	Montana	10	5
2.	*viridis*	Wyoming	14	7
3.	*viridis*	Colorado, Nebraska, Kansas, Oklahoma, South Dakota, Texas	21	23
4.	*viridis-nuntius*	New Mexico	19	16
5.	*nuntius*	Arizona (Coconino, Navajo, Apache, Gila Counties)	37	15
6.	*abyssus*	Arizona (Coconino, Mohave Counties)	6	6
7.	*cerberus*	Arizona (Central Highlands)	20	8
8.	*cerberus*	Arizona (Pinaleño-Galiuro-Santa Catalina Mountains)	12	3
9.	*concolor*	Utah (east)	13	6
10.	*concolor*	Wyoming (Sweetwater)	4	5
11.	*lutosus*	Utah (west); Nevada	19	15
12.	*lutosus*	Nevada (Anahoe Island)	9	7
13.	*lutosus*	California (Mono, Lassen, Modoc)	7	0
14.	*helleri*	California (San Bernardino, Riverside, San Diego Counties; Mexico, Baja California)	19	13
15.	*helleri*	California (Santa Barbara, Ventura, Los Angeles Counties)	10	7
16.	*caliginis*	Mexico, Isla Coronado Sur, Los Coronados	9	10
17.	*oreganus*	California (Merced, Madera, Fresno, Tulare, El Dorado, Kern Counties)	12	8
18.	intermediate	California (Monterey, San Luis Obispo Counties)	9	9
19.	*oreganus*	California (Santa Cruz, Santa Clara, San Benito, San Mateo Counties)	12	4
20.	*oreganus*	California (Butte, Plumas, Tuolumne Counties)	3	2
21.	*oreganus*	California (Siskiyou, Humboldt, Sonoma, Trinity, Alameda, Contra Costa, Stanislaus, Lake, Mendocino Counties)	8	15
22.	*oreganus*	Canada (British Columbia); Washington; Oregon	8	14
23.	*lutosus*	Idaho (Nez Perce, Latah Counties)	6	1
24.	intermediate	Idaho (Adams, Washington Counties)	11	0
25.	*lutosus*	Idaho (Ada, Canyon, Elmore Counties)	10	10
26.	*concolor*?	Utah (Carbon)	2	0
27.	*concolor*?	Utah (Garfield)	4	0

The final choices of OTUs (Table 3.2 and figure 3.2) were compared against Mahalanobis distance matrices generated in BMDP 7M which highlights individuals

for which the D^2 value is closer to another group than that to which it has been assigned. Overall the Mahalanobis matrix conformed to OTU composition, with the exception of one or two individuals from very closely related groups, for example one from OTU 2 (Wyoming) which might have been better placed in OTU 1, Montana. These "conflicts" were considered to be trivial (due to the extremely close morphological similarity of snakes from these areas), and OTU structure was therefore not changed.

3.3.2 Characters

All meristic and mensural characters showed low pooled within sample correlation (calculated from a pooled within-groups covariance matrix provided in the BMDP AM output).

3.3.2.i *Meristic characters*

All meristic characters (n=33) examined by 2-way ANOVA (Table 3.3) were highly significant with respect to variation between OTUs (localities). Of these characters 18 (54.54%) were sexually dimorphic, in particular characters relating to the posterior body and tail. Eleven characters (33.33%; predominantly pattern) were found to be significant for the interaction between locality and sex.

3.3.2.ii *Mensural characters*

Choice of log transformation model varied for both character and sex. Two-way ANCOVA revealed 25 characters of which 24 (96%) varied significantly with locality, 19 (76%) were sexually dimorphic, and only 2 (8%) varied with locality and sex together (Table 3.4). One-way ANCOVA revealed 23 characters were significantly

geographically variable among males, and 21 among females (Table 3.4). Note that fang length was excluded from these analyses due to excess missing data.

Table 3.3. *F*-values of 2-way ANOVA showing which meristic morphological characters show significant variation between 1) geographical locality (group), 2) sex, and/or 3) interaction between locality and sex. Significant characters are marked as follows: $P<0.01$**, $P<0.05$*.

Character	Group	Sex	Group/ Sex
Lepidosis			
No. dorsal scale rows at 10% of body length	4.47 **	0.69	0.68
No. dorsal scale rows at 20% of body length	6.05 **	0.44	0.71
No. dorsal scale rows at 30% of body length	4.17 **	0.00	0.77
No. dorsal scale rows at 40% of body length	5.50 **	0.55	1.30
No. dorsal scale rows at 50% of body length	6.22 **	0.02	1.44
No. dorsal scale rows at 60% of body length	6.77 **	0.67	0.54
No. dorsal scale rows at 70% of body length	5.69 **	5.52 *	0.95
No. dorsal scale rows at 80% of body length	9.03 **	4.32 *	1.58 *
No. dorsal scale rows at 90% of body length	10.69 **	11.20 **	0.92
No. dorsal scale rows at 100% of body length	9.15 **	4.34 *	0.96
No. dorsal scale rows at 10% of tail length	12.38 **	19.26 **	0.87
No. dorsal scale rows at 25% of tail length	7.38 **	5.86 *	0.77
No. dorsal scale rows at 75% of tail length	9.60 **	11.05 **	1.56
No. ventral scales	8.24 **	59.44 **	0.80
No. subcaudal scales	3.74 **	760.77 **	1.06
No. supralabials	4.30 **	6.51 *	0.87
No. infralabials	2.70 **	1.88	0.55
No. intersupraoculars	22.00 **	1.88	1.58 *
No. interoculabials	5.01 **	6.25 *	0.87
No. prefoveals	2.89 **	6.44 *	0.80
General pattern			
No. dorsal blotches	27.71 **	2.30	2.14 **
No. tail bands	10.80 **	29.08 **	0.91
% dorsal blotches fused with lateral blotches	9.63 **	2.88	0.95
Amount of ventral mottling	17.77 **	0.15	0.48
Mean length of blotch edge at 25%	2.34 **	3.09	1.31
Mean length of blotch edge at 50%	12.91 **	4.67 *	2.07 **
Mean length of blotch edge at 75%	12.32 **	3.66	1.86 **
Mean blotch midline length at 25%	16.68 **	4.21 *	1.75 *
Mean blotch midline length at 50%	22.82 **	7.80 **	2.22 **
Mean blotch midline length at 75%	28.77 **	1.35	2.16 **
Blotch width at 25%	5.53 **	5.58 *	2.54 **
Blotch width at 50%	4.53 **	6.33 *	2.16 **
Blotch width at 75%	14.51 **	3.21	2.66 **

Table 3.4. *F*-values of 2-way ANCOVA showing which mensural characters are signficantly variable between 1) geographical locality (group), 2) sex, and/or 3) interaction between locality and sex. Significant characters are marked as follows: $P<0.01$**, $P<0.05$*.

Character	Group	Sex	Group/ Sex
Tail length	2.50**	425.06**	2.00**
Proximal rattle depth	17.34**	18.08**	1.17
Proximal rattle length	5.80**	6.73**	0.69
Proximal rattle width	9.28**	12.31**	1.08
Head length (quadrate tip to snout tip)	2.73**	2.13**	1.37
Head width at widest point (gape)	6.28**	6.73	0.74
Head width at eye level	3.57**	14.58**	1.17
Snout width (immediately anterior to eyes)	1.89**	16.47**	1.27
Snout length	4.74**	12.03**	1.08
Distance between eye and nostril	2.30**	5.49*	0.53
Distance between eye and pit	2.47**	5.13*	0.70
Distance between pit and nostril	3.79**	3.70	0.94
Eye diameter	3.32**	8.85**	0.44
Pit diameter	3.12**	0.64	0.75
Mental scale length	1.54	5.04*	0.55
Mental scale width	2.91**	32.19**	0.81
Rostral scale length	8.04**	7.66**	1.32
Rostral scale width	2.85**	8.79**	0.86
Prenasal scale length	4.10**	28.33**	0.59
Prenasal scale width	4.51**	13.33**	1.23
Supraocular scale length	3.70**	3.40	0.56
Supraocular scale width	5.37**	9.89**	1.22
Length of dorsal blotches at 25%	15.64**	6.71**	0.77
Length of dorsal blotches at 50%	24.29**	0.30	2.44**
Length of dorsal blotches at 75%	34.66**	1.01	1.02

Table 3.5. Results of 1-way ANCOVA of mensural characters with size (SVL) for (a) males and (b) females. For all characters the probabilities of adjusted means are significantly different ($P<0.05$) and there is no significant difference in the equality of regression slopes ($P>0.05$). Characters not fitting to these significance criteria (also see methods) were rejected. The regression models used include: no transformation of either character or SVL (-); log transformation of SVL only (†), or log transformation of both character and SVL (*).

	Character	Regression model	Regression coefficient	Standard error	Adjusted means *F*-value	Equality of slopes *F*-value
(a)	**Body dimensions**					
	Tail length	†	90.050	2.915	2.169	1.185
	Head length	-	0.035	0.001	2.277	0.922
	Head width (widest point)	*	0.754	0.025	4.819	1.229
	Snout length	*	0.616	0.017	3.261	0.882
	Distance: eye to nostril	*	0.677	0.030	1.728	0.400
	Distance: eye to pit	*	0.723	0.030	1.633	0.721
	Distance: nostril to pit	*	0.709	0.028	3.366	0.532
	General pattern					
	Blotch length at 25% (mm)	*	0.869	0.067	7.761	1.470
	Blotch length at 50% (mm)	*	1.064	0.564	13.966	0.468
	Blotch length at 75% (mm)	*	1.063	0.065	15.887	0.636
	Other dimensions					
	Proximal rattle length	*	0.815	0.036	7.130	0.907
	Proximal rattle depth	†	17.226	0.660	7.732	0.863
	Proximal rattle width	-	0.006	0.000	7.508	1.546
	Eye diameter	*	0.472	0.026	2.168	1.532
	Pit diameter	*	0.520	0.030	2.231	1.137
	Mental scale width	-	0.005	0.000	2.081	0.859
	Rostral scale length	*	0.614	0.023	6.075	0.766
	Rostral scale width	-	0.004	0.000	2.770	1.010
	Prenasal scale length	*	0.703	0.027	2.920	1.392
	Prenasal scale width	*	0.654	0.026	3.430	0.806
	Supraocular scale length	†	9.682	0.499	1.949	1.511
	Supraocular scale width	*	0.648	0.020	4.435	0.968
	Fang length	†	13.335	0.767	6.046	1.254
(b)	**Body dimensions**					
	Widest head width	*	0.745	0.035	3.618	1.088
	Head width between eyes	*	0.599	0.026	2.015	0.716
	Snout length	*	0.591	0.022	3.134	1.055
	Distance: eye to pit	*	0.674	0.037	1.910	0.468
	Distance: nostril to pit	*	0.662	0.039	2.677	1.186
	General pattern					
	Blotch length at 25% (mm)	*	0.829	0.066	10.074	0.543
	Blotch length at 50% (mm)	*	1.003	0.093	7.318	1.476
	Blotch length at 75% (mm)	*	0.996	0.067	16.318	0.847
	Other dimensions					
	Proximal rattle length	*	0.256	0.045	2.909	0.733
	Proximal rattle depth	*	0.757	0.029	6.600	0.484
	Eye diameter	*	0.500	0.030	2.231	1.168
	Pit diameter	*	0.532	0.032	2.307	1.441
	Mental scale length	*	0.590	0.036	1.809	0.760
	Mental scale width	*	0.632	0.347	2.374	1.409
	Rostral scale length	*	0.545	0.028	4.805	0.718
	Rostral scale width	*	0.643	0.034	2.291	0.738
	Prenasal scale length	*	0.635	0.040	2.254	0.714
	Prenasal scale width	*	0.628	0.031	3.475	0.925
	Supraocular scale length	*	0.564	0.034	1.972	1.071
	Supraocular scale width	-	0.012	0.001	3.090	0.996
	Fang length	*	0.791	0.046	2.695	1.124

3.3.3 Patterns of geographical variation.

Canonical variate analysis did not reveal any discrete groups corresponding to any one of the nine subspecies categories. Three large, loosely defined groups are apparent, relating to the major physiographic regions south and east of the Rocky Mountains (SER - including *C. v. viridis* and *C. v. nuntius*), the Pacific Coast (PC - including *C. v. helleri, C. v. oreganus*, and *C. v. caliginis*) and the Great Basin (GB - including *C. v. abyssus* and *C. v. lutosus*) although there is overlap between the PC and GB individuals in the males, and the SER and GB individuals in the females (figure 3.3). Figure 3.4 illustrates generalised plots of the mean canonical variate scores for each sex, and the regional categories. The numbers represent the locality labels as defined by PCA (Table 3.2).

The position of certain groups is unclear, and inconsistent between sexes. In the males the two central and southern Arizona groups (*C. v. cerberus*: 7 and 8) fall between the PC and GB, locality 10 (*C. v. concolor*: Sweetwater County, Wyoming) in the GB region, and the Colorado Plateau (CP) individuals in Utah (*C. v. concolor*: 9, 26, and 27) between and partially overlapping with GB and SER. In the females locality 7 forms an almost independent group (note: group 8 only consists of males), locality 10 lies between the PC and SER areas respectively, and individuals from 9 are continuous with those from the SER region (there are no females from localities 26 and 27).

High (standardised) first canonical variate coefficients in males are most heavily associated with a high number of tail bands, a low intersupraocular scale count, and short dorsal blotches on the lower third of the body, and to a lesser extent with a low dorsal scale row count 10% along the body (i.e. the position at 10% along of the total number of ventral scales), and a low number of fused dorsal blotches. High second canonical variate coefficients in males are most closely associated with a low intersupraocular scale count, less diamond-like dorsal blotches on the lower third of

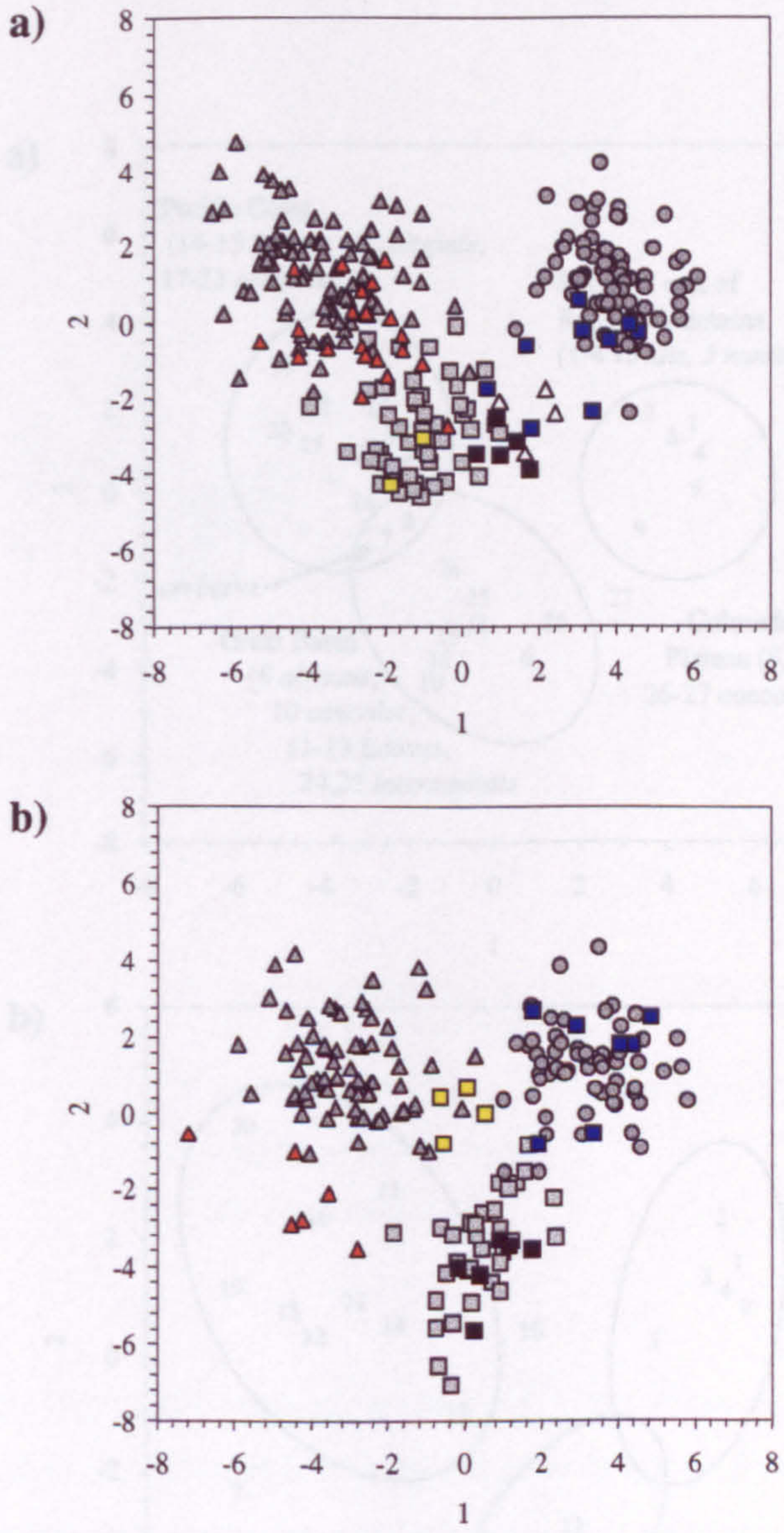


Figure 3.3. Ordination based on all significantly variable characters along the first two canonical variates in **a)** males and **b)** females. The first and second canonical variates summarise respectively 41.76% and 11.00% and 33.01% and 17.43% of total variance. When *C. v. cerberus* and *C. v. concolor* are excluded from the analysis, the pattern of separation is unaffected.

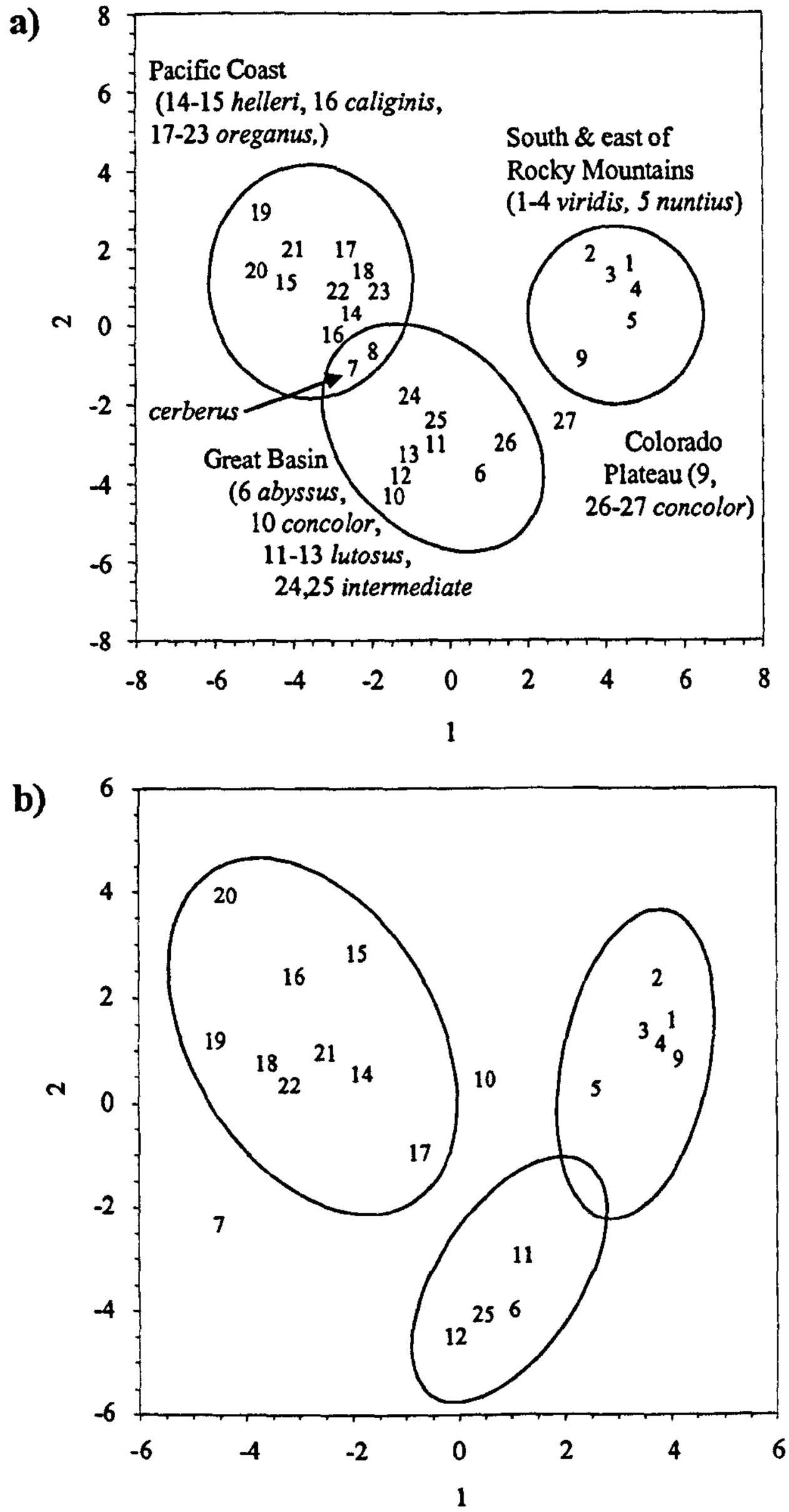


Figure 3.4. Ordination of group means for all significantly variable characters along the first two canonical variates in **a)** males and **b)** females. Circles are used to illustrate the generalised regional pattern. When *cerberus* and *concolor* are excluded from the analysis, the pattern of separation is unaffected.

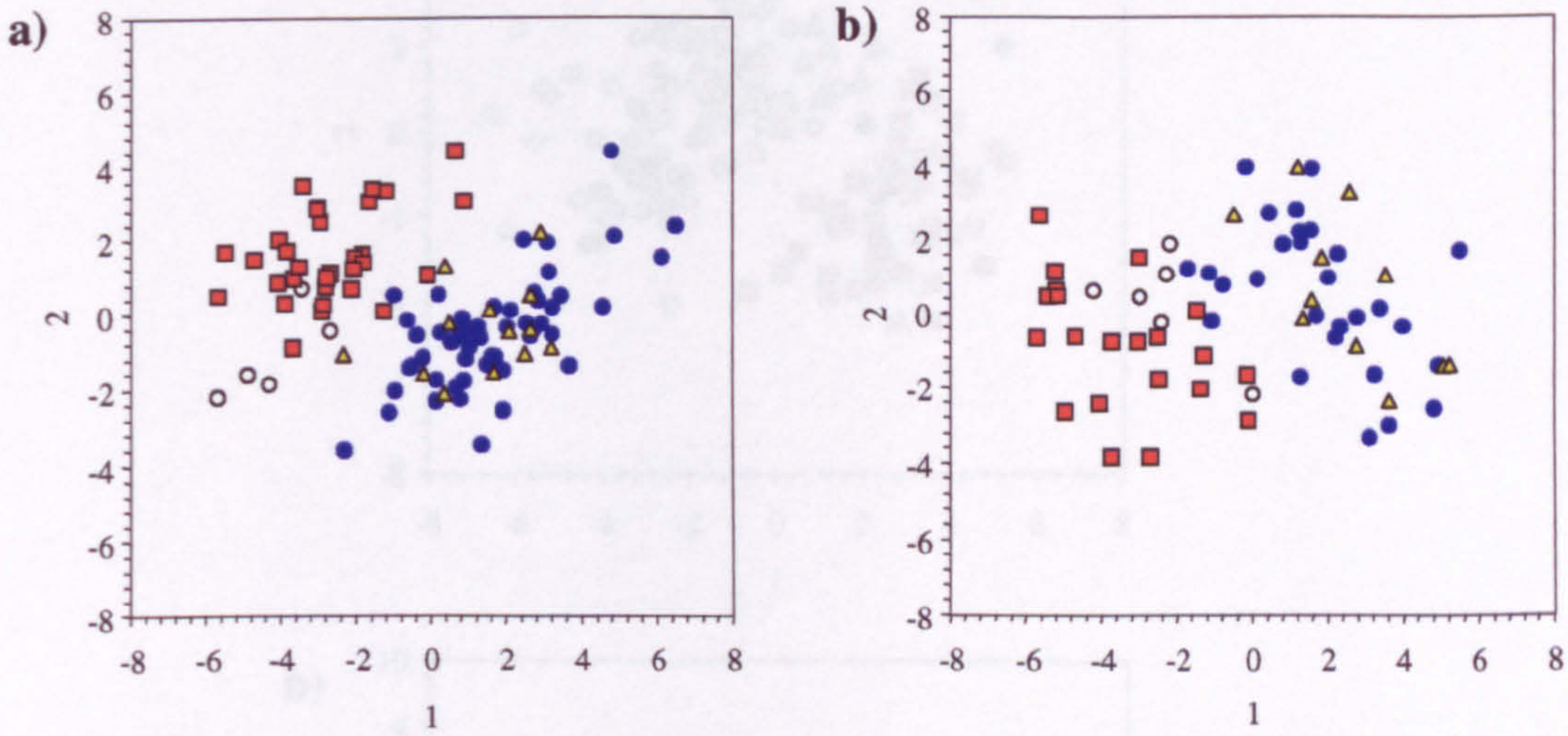


Figure 3.5. Geographic variation between the Great Basin region and populations east-south of the Rocky Mountains in **a**) males and **b**) females. The scatter plot of the first two principal component scores for significantly variable characters, shows a clear separation between the Great Basin and Grand Canyon forms (*abyssus/lutosus*) and the east/south Rockies forms (*viridis/nuntius*). Rather than forming an intermediate, *C. v. concolor* groups with the eastern forms. In males and females respectively, the first principal component describes 22.2% and 28.4% of the variation, and the second 9.7% and 10.6%.

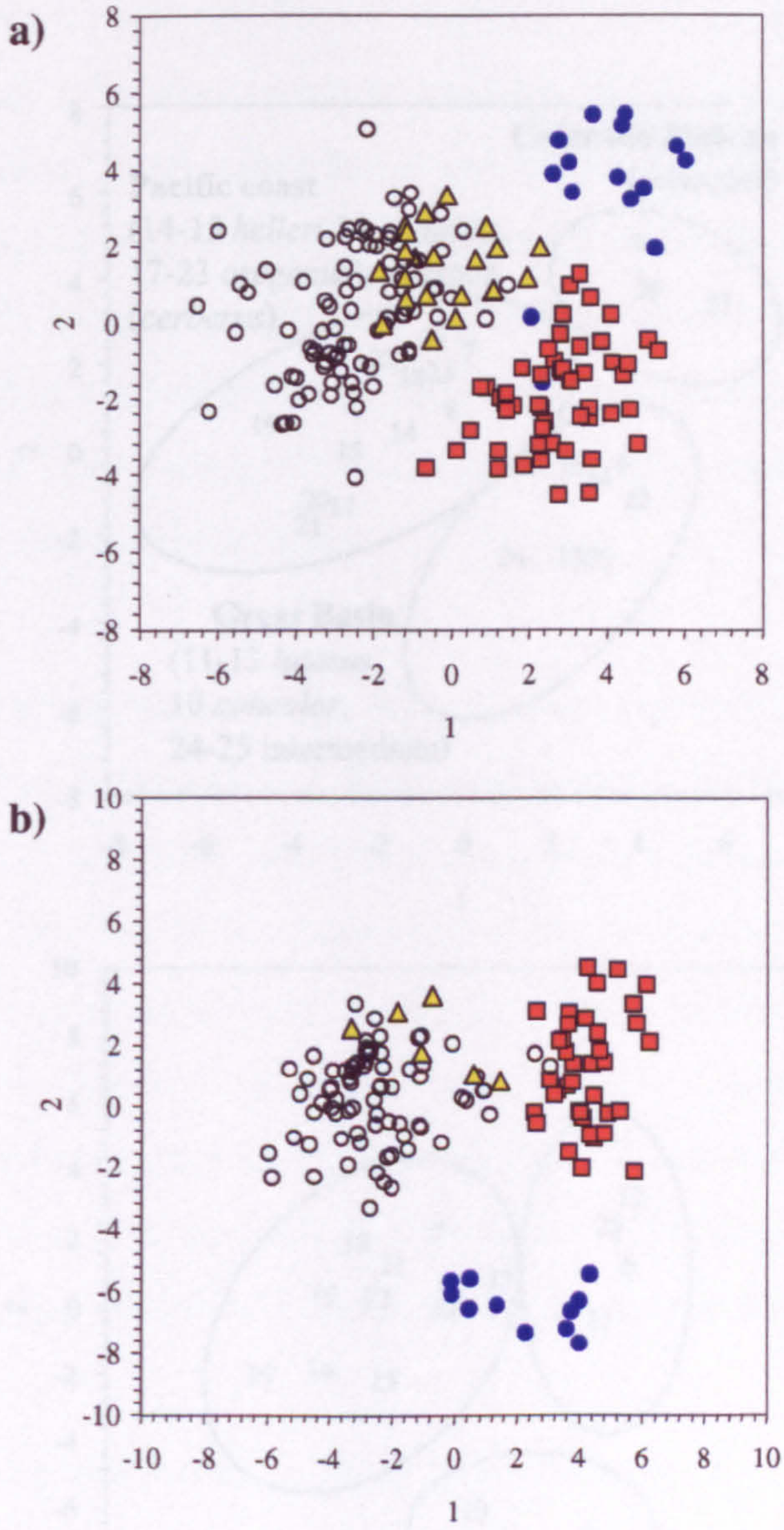
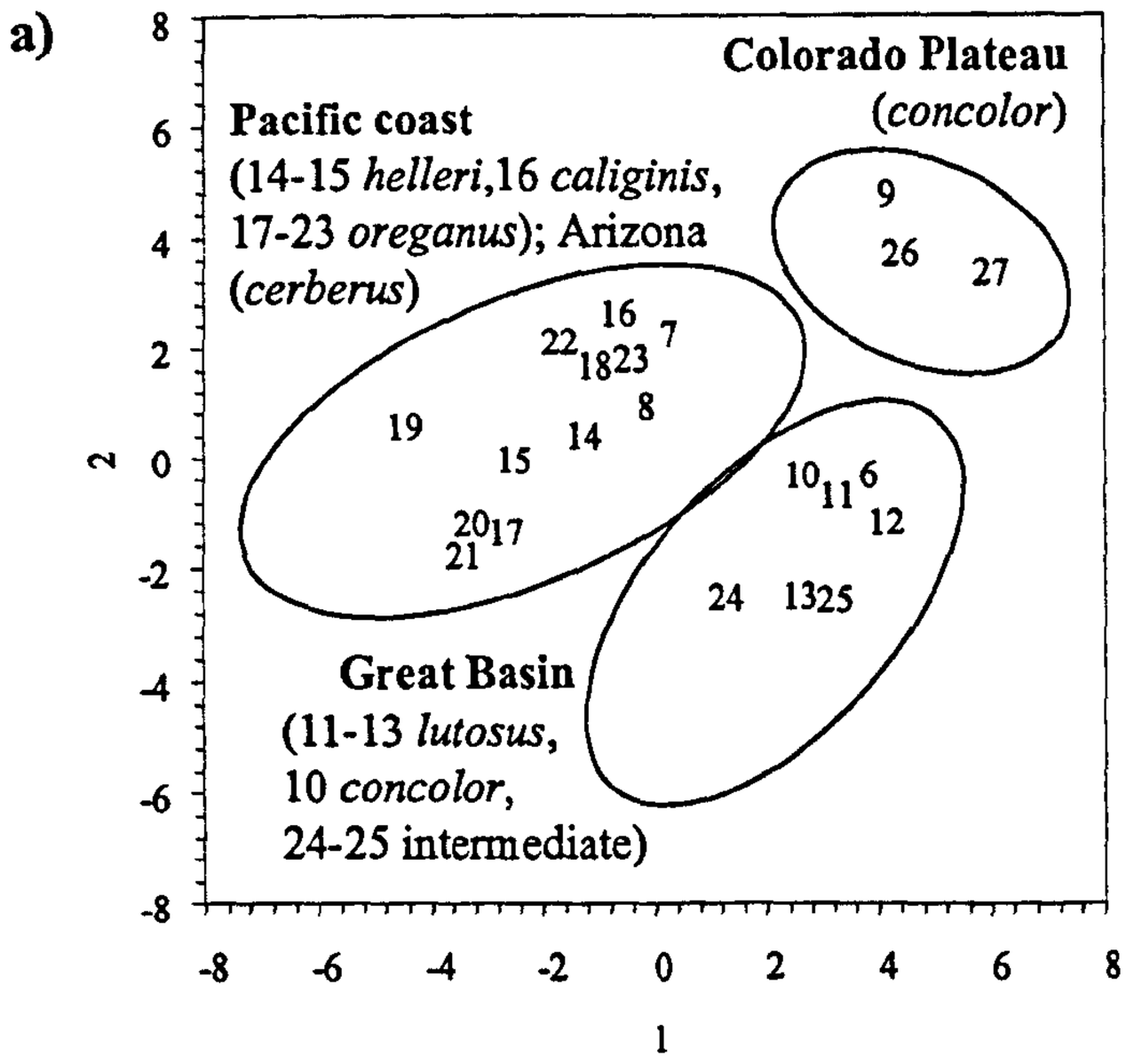


Figure 3.6. Ordination of all significantly variable characters along the first two canonical variates in **a)** males and **b)** females occurring west of the Rocky Mountains. The first and second canonical variates summarise respectively 27.03% and 13.92% and 35.14% and 17.94% of the total variance.



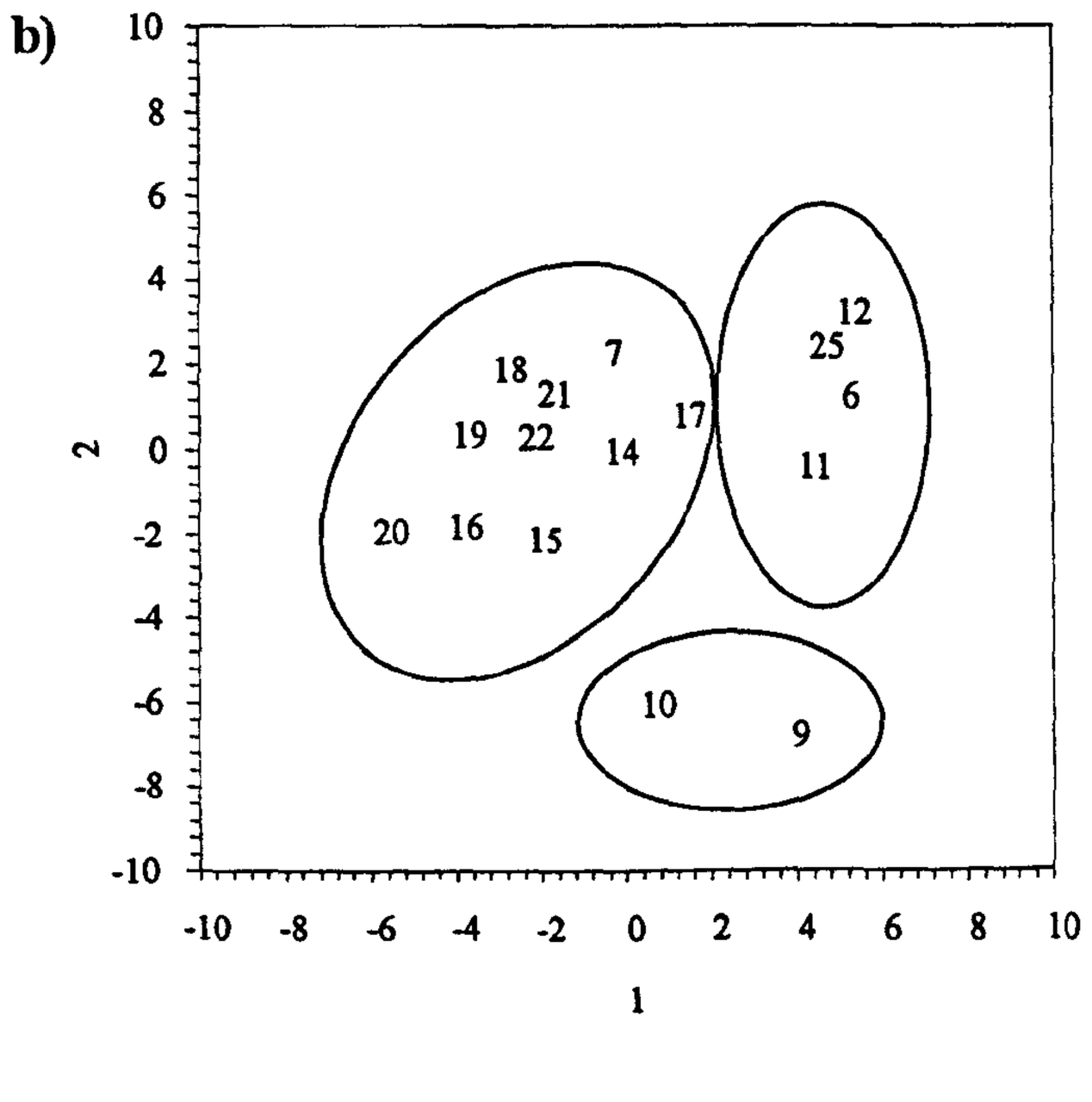


Figure 3.7. Ordination of group means for all populations occurring west of the Rocky Mountains along the first two canonical variates in **a)** males and **b)** females. Circles represent the generalised regional pattern.

the body, and larger rattle size (or tail depth). In females, high coefficients are most closely associated with smaller rattles (narrower tails) and shorter blotches at mid-body, and to a lesser extent with a low dorsal scale row count at the 90% VS position, and a greater length between eye and pit. High scores for the second canonical variate are most closely associated with low intersupraocular scale count, longer dorsal blotches on the first third of the body, and high dorsal scale count at the 10% position. In all analyses the third canonical variate (and principal component) did not contribute in elucidating the populations further.

A CVA on the eastern cluster alone did not reveal any further population structuring. The position of male populations from south-eastern Utah (localities 9, 26, 27) in relation to the GB and SER populations, suggests a potentially clinal pattern from south and east of the Rocky Mountains across the Colorado Plateau to the Great Basin (figure 3.3.a). However a PCA of significantly variable characters between the GB and SER populations (figure 3.5) shows that, in both males and females, the CP populations do not represent an intermediate form between GB and SER. Rather, the CP individuals tend to group with or close to the SER populations. Plotting the third against first principal component scores does not help to elucidate the pattern.

Ordination of all populations occurring west of the Rocky Mountains (i.e. excluding localities 1-5) shows more marked separation of the PC and GB regional groups, however the Colorado Plateau populations now form an independent group away from either the Pacific Coast or Great Basin, in both males and females (figures 3.6 and 3.7). The remaining conventional *C. v. concolor* population, 10 (Sweetwater County, Wyoming) remains closely grouped with the GB populations in the males, but joins the other CP population, 9, in the females. In both sexes *C. v. cerberus* groups with the PC forms rather than forming a discrete entity.

In males, high canonical variate coefficients were largely associated with large numbers of dorsal blotches, more diamond-shaped dorsal blotches on the lower third of the body, and to a lesser extent with low numbers of dorsal blotches fused with

lateral and reduced rattle size (tail thickness). High values for the second canonical variate were associated mostly with more diamond-shaped dorsal blotches from mid to the lower third of the body, and low numbers of ventral and infralabials. With respect to females, high values were very highly associated with more diamond shaped dorsal blotches on the first third of the body, and to a lesser extent with wide distal dorsal blotches, smaller rattle size (thinner tail), and high scale counts at the 10% VS position. The second canonical variate was most heavily associated with a low dorsal scale count at the 40% VS position, a larger rattle with respect to depth (therefore thicker tail), and large number of intersupraocular scales.

3.3.4 Contact zones

3.3.4.i *Californian transect*

The scatter plots of the mean CVA scores in figure 3.4 shows that the Pacific Coastal forms (southern and northern Californian, Washington, and Idaho individuals) appear to form a fairly continuous, mixed population. Further clarification of the relationships of these populations is not achieved by canonical variate analysis in either males or females. When the Pacific Coast groups are analysed exclusively the individual populations completely depart from each other to form discrete entities, hence failing to provide any useful information.

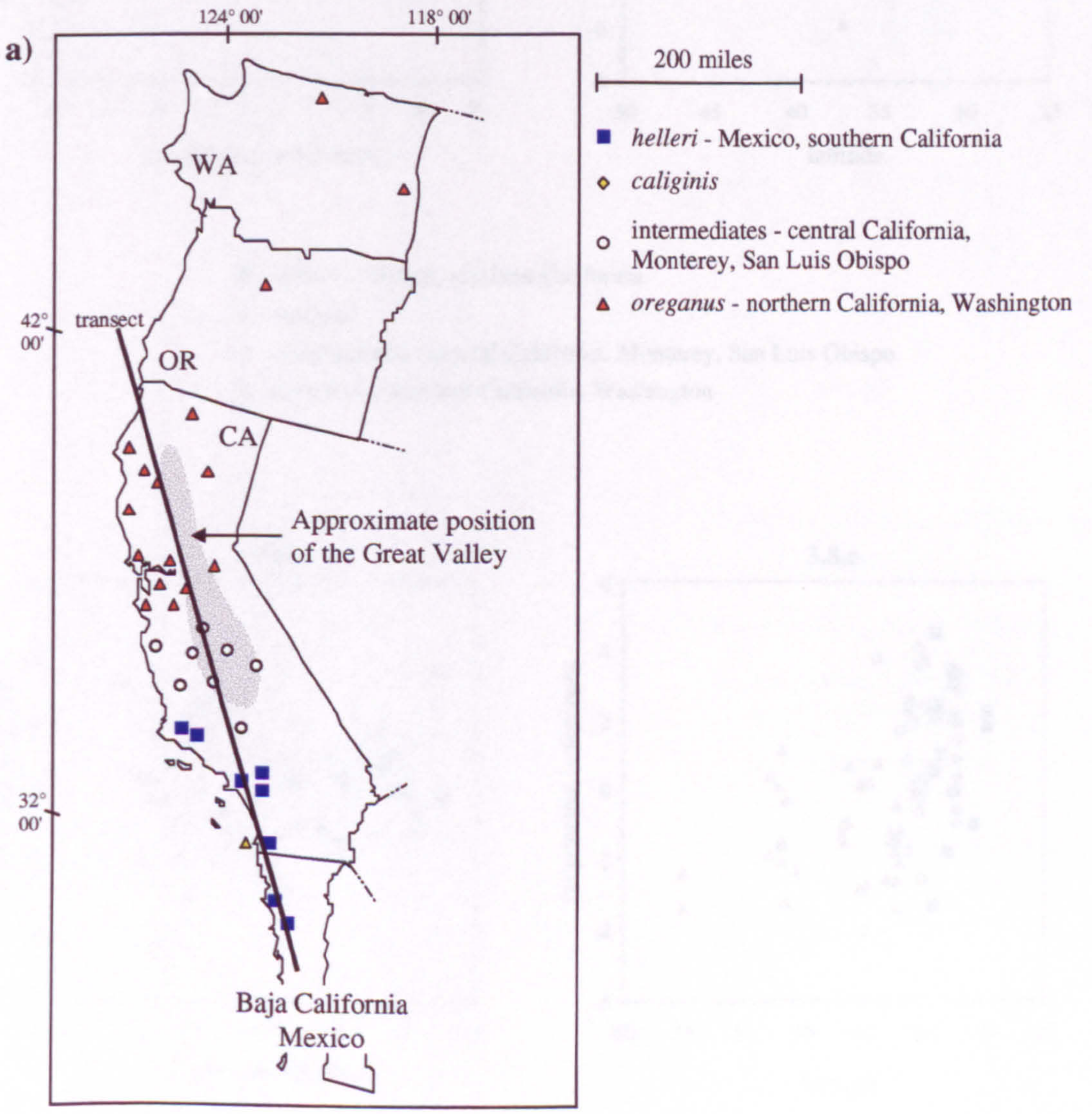
Principal component analysis of significantly variable characters for the coastal Pacific region males, however, suggests a gradual change from the extreme northern *C. v. oreganus*, through Monterey, San Luis Obispo, and the southern Central Valley counties, to the southern *C. v. helleri* and *C. v. caliginis* populations (figure 3.8.b). The transect plot of the first principal component against latitude (figure 3.8.c) further suggests a north-south clinal pattern in the form of an increasing principal component score moving southwards. The geographical position of the clinal pattern is illustrated in (figure 3.8.a). A similar, although less pronounced pattern is also seen in the

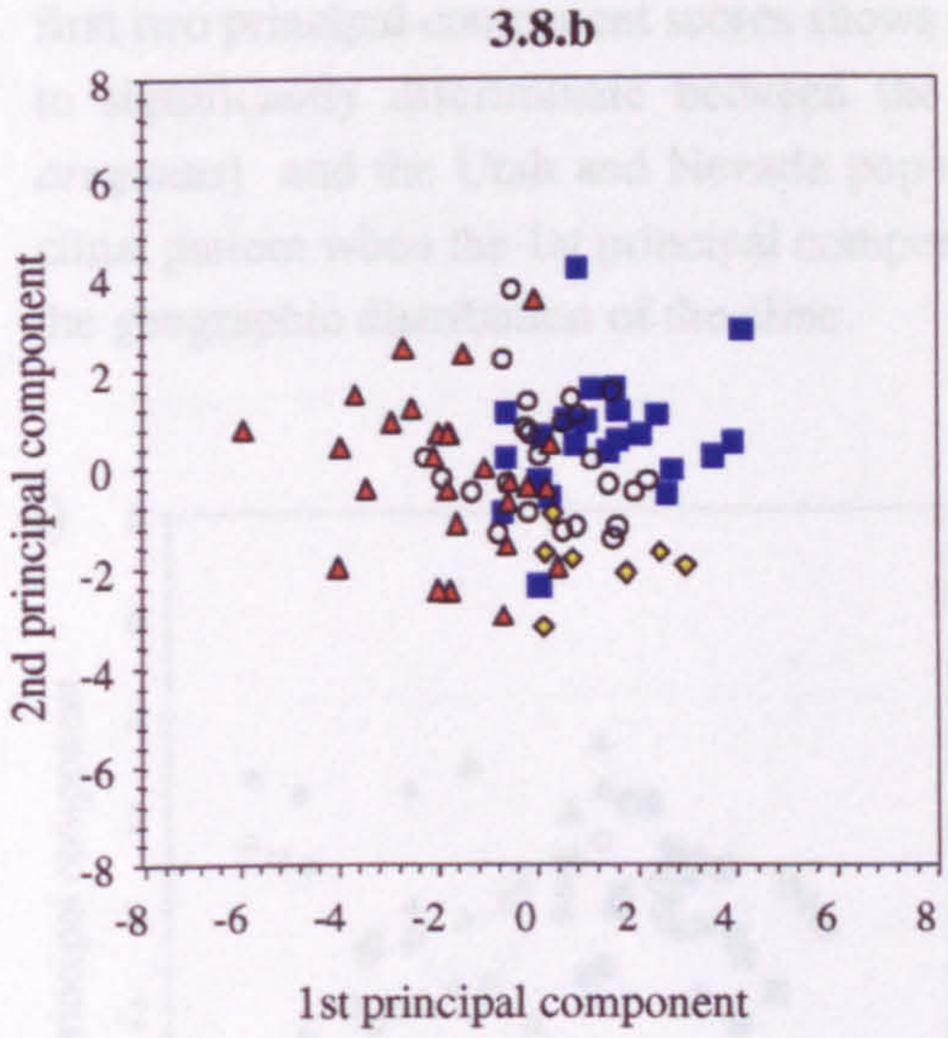
females (figures 3.8d and 3.8e). A Spearman's product moment correlation between first principal component score and latitude shows that the clinal relationship is highly significant (correlation coefficient = -0.385; $P<0.01$) in both sexes.

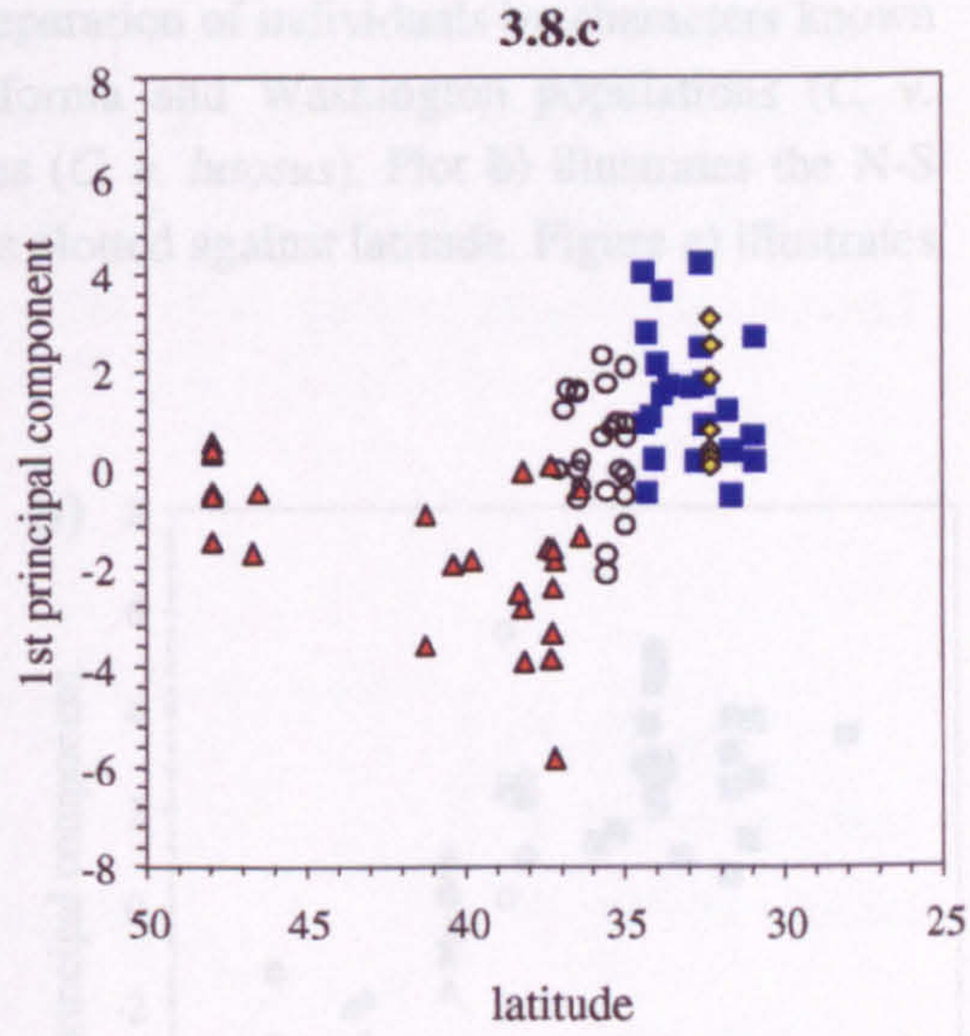
3.3.4.ii *Idaho transect*

In the CVA plots of the means for all localities (figure 3.4.) and west of the Rocky Mountains (figures 3.7) groups 24 and 25 (Ada/Elmore and Adams Counties, Idaho) lie with the other Great Basin localities, whilst group 23 (Nez Perce and Latah Counties, Idaho) falls within in the Pacific Coast regional group. The same trend is shown in the PCA plot in figure 3.9.a, of the first against second principal component for characters significant just to the localities under consideration, however the Adams County, Idaho individuals appear more intermediate between the Great Basin and Pacific Coast individuals, as opposed to grouping more closely with the GB individuals. When the first principal component is plotted against latitude (figure 3.9.b) a north-south clinal trend is apparent, with a decrease in principal component score moving northwards. The black diamond symbols illustrate how the first principal component scores of *C. v. oreganus* populations are constant against latitude, but change moving towards the Great Basin *C. v. lutosus*. The geographical position of the trend, which appears to correspond to the course of the Snake River, is shown on the map in figure 3.9.c. A product moment correlation (conducted as for the California transect) of individuals along the Idaho transect, and excluding the northern California individuals, suggests that the clinal relationship is highly significant (correlation coefficient = -0.461; $P<0.01$).

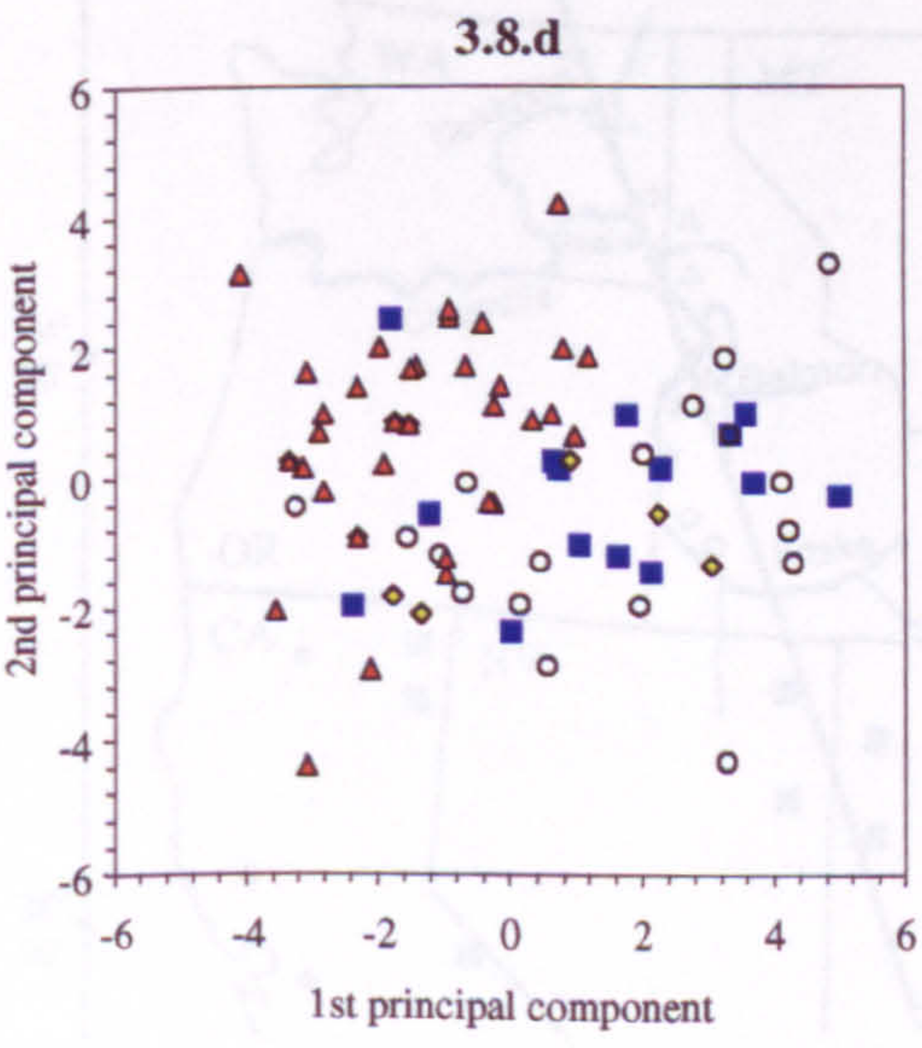
Figure 3.8. North-south clinal variation between *C. v. oreganus* and *C. v. helleri* along a transect in the Pacific Coastal region, from Washington to Mexico (**a**). For males and females respectively, **b & d**) scatter plots of the first two principal components; **c & e**) show the relationship of first principal component score with latitude. (**b**) illustrates the distinct northerly and southerly (including South Island, Los Coronados) populations of Pacific rattlesnake with intermediate forms from central California (Fresno, Kern, Merced, Madera, Tulare, and El Dorado Counties) and the supposed contact zone between the conventional subspecies, Monterey and San Luis Obispo. Plot (**c**) clearly (and plot (**e**). to a lesser extent) illustrates the gradual change in 1st principal component scores with latitude, again depicting distinct north and south populations with intermediate forms.







■ *helleri* - Mexico, southern California
◆ *caligini*s
○ intermediates - central California, Monterey, San Luis Obispo
▲ *oreganus* - northern California, Washington



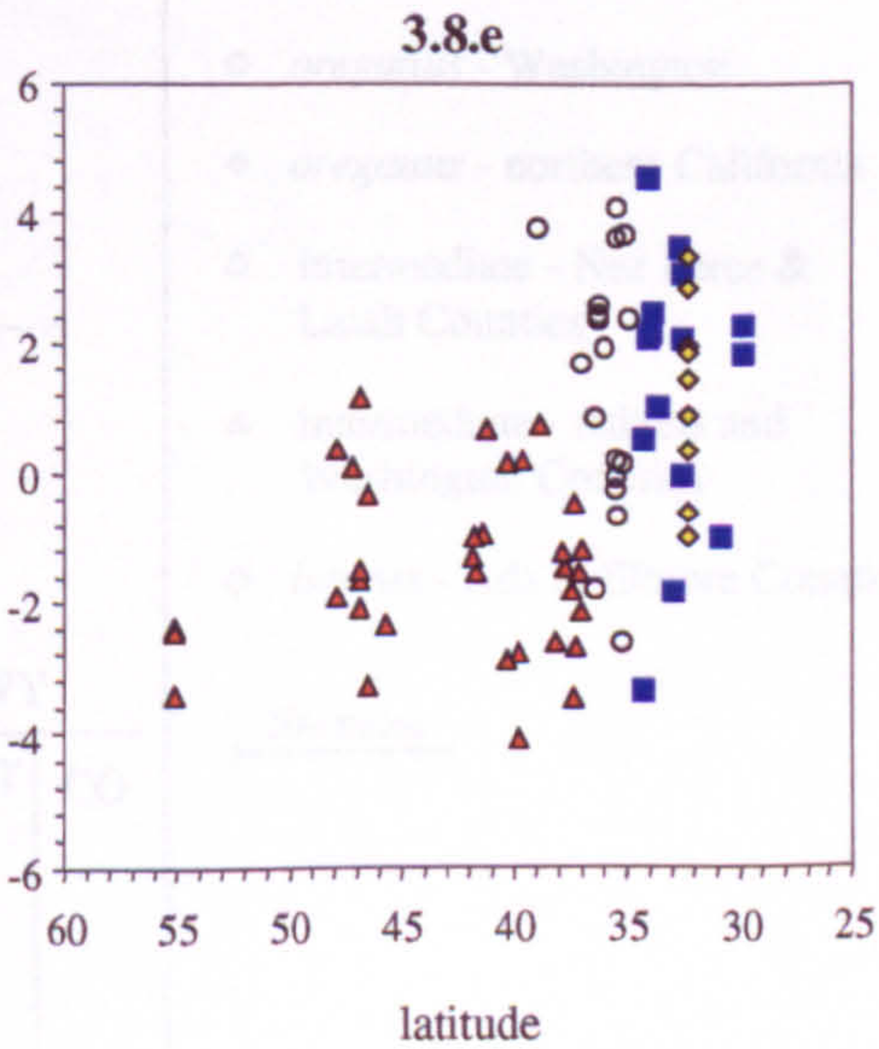
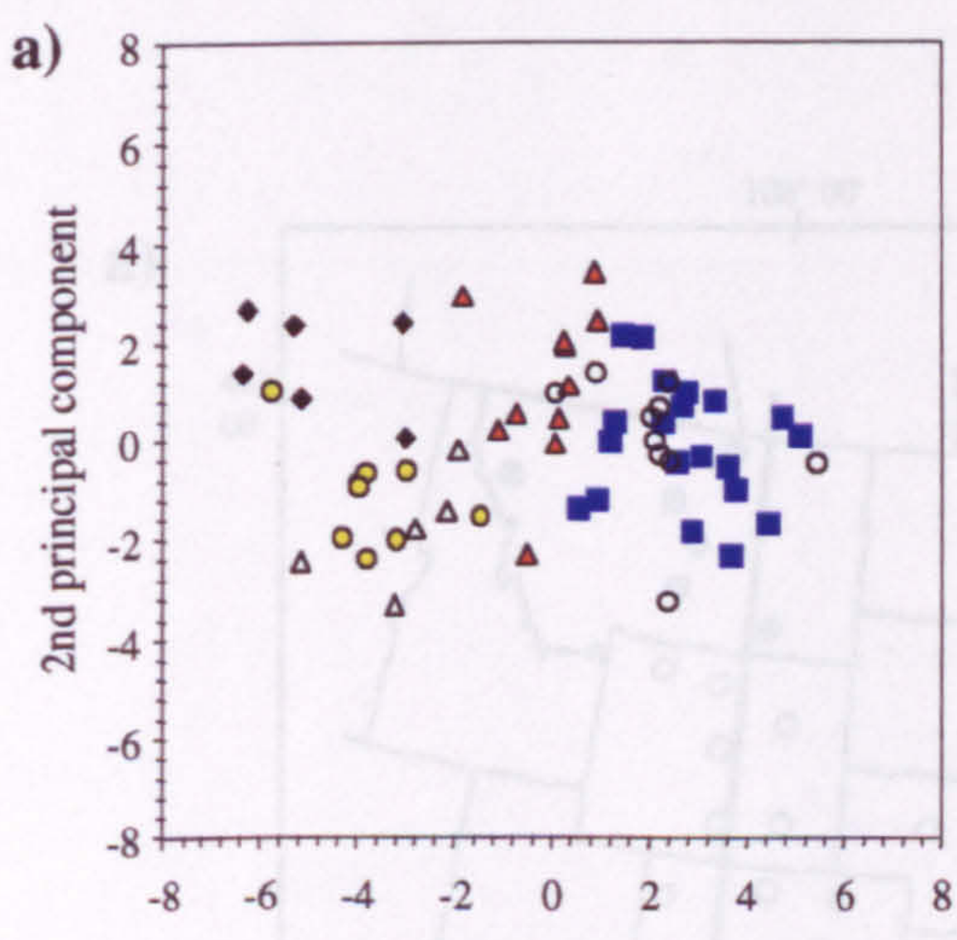
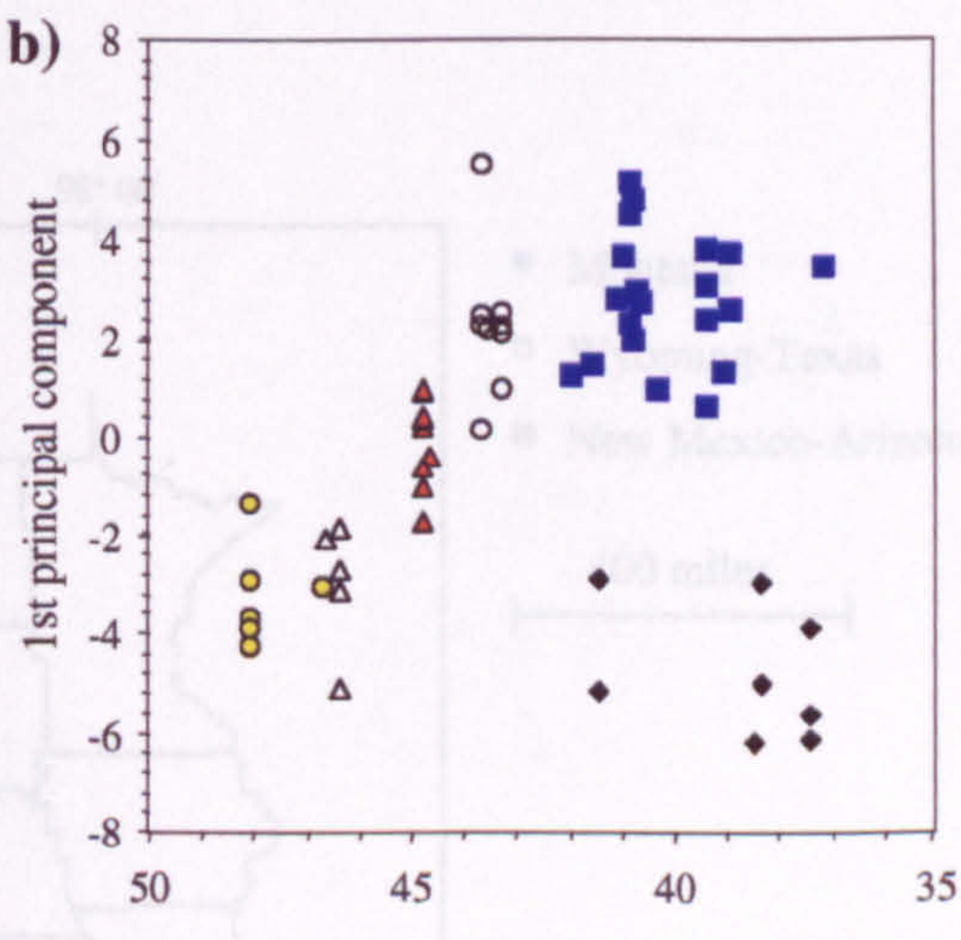


Figure 3.9. Clinal variation in male *Crotalus viridis oreganus* and *C. v. lutosus* from Washington State, south through Idaho, to Utah and Nevada. Note that females are not compared due to a lack of samples from the intermediate localities. The scatter plot **a**) of the first two principal component scores shows the separation of individuals by characters known to significantly discriminate between the California and Washington populations (*C. v. oreganus*) and the Utah and Nevada populations (*C. v. lutosus*). Plot **b**) illustrates the N-S clinal pattern when the 1st principal component is plotted against latitude. Figure **c**) illustrates the geographic distribution of the cline.





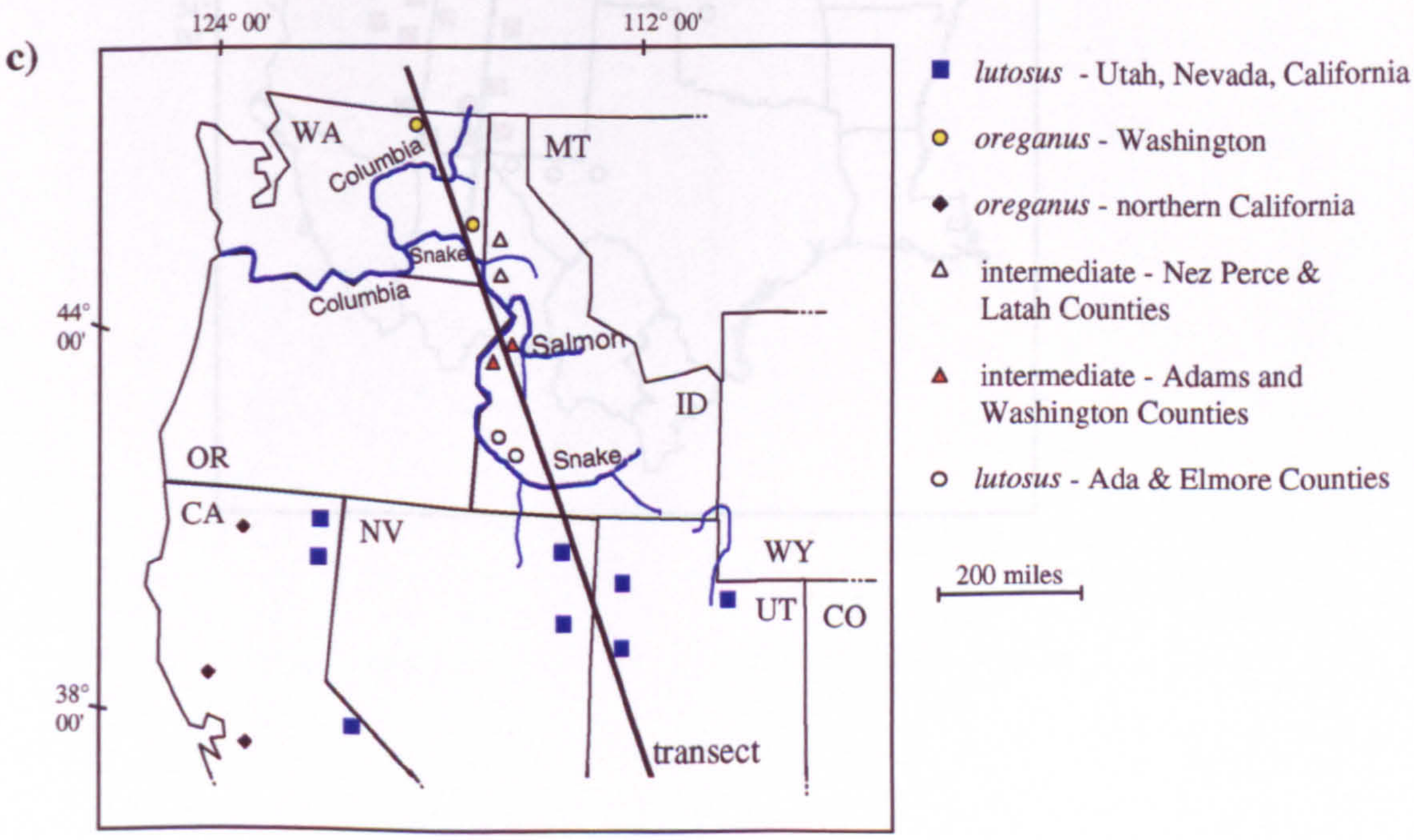
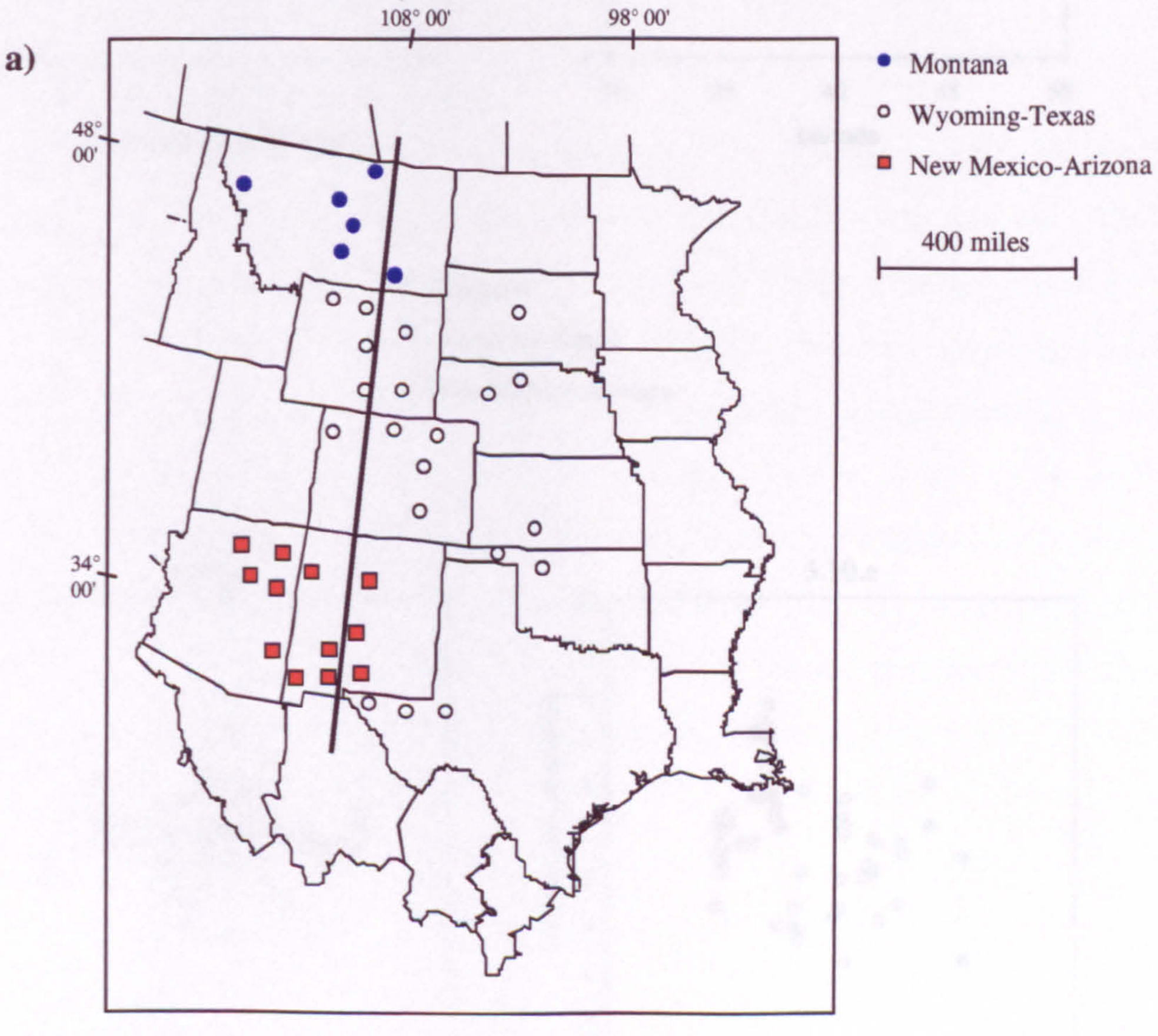
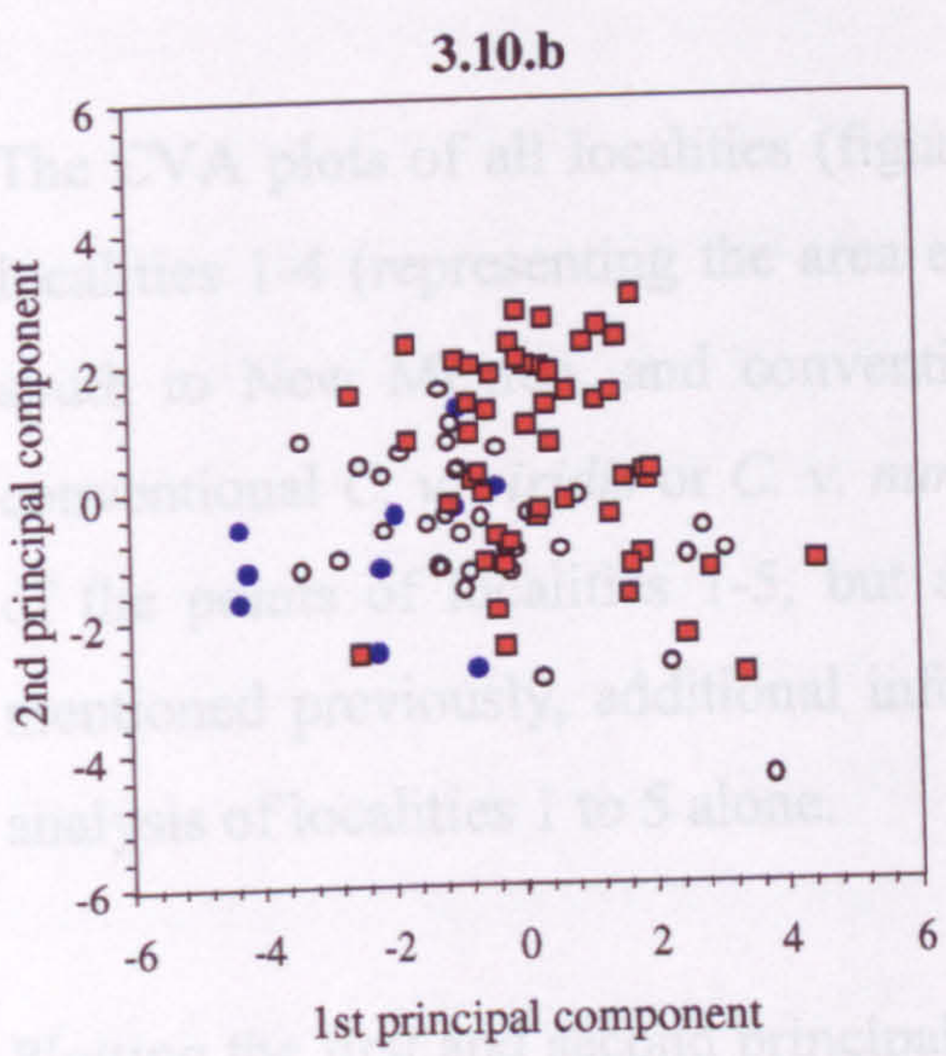
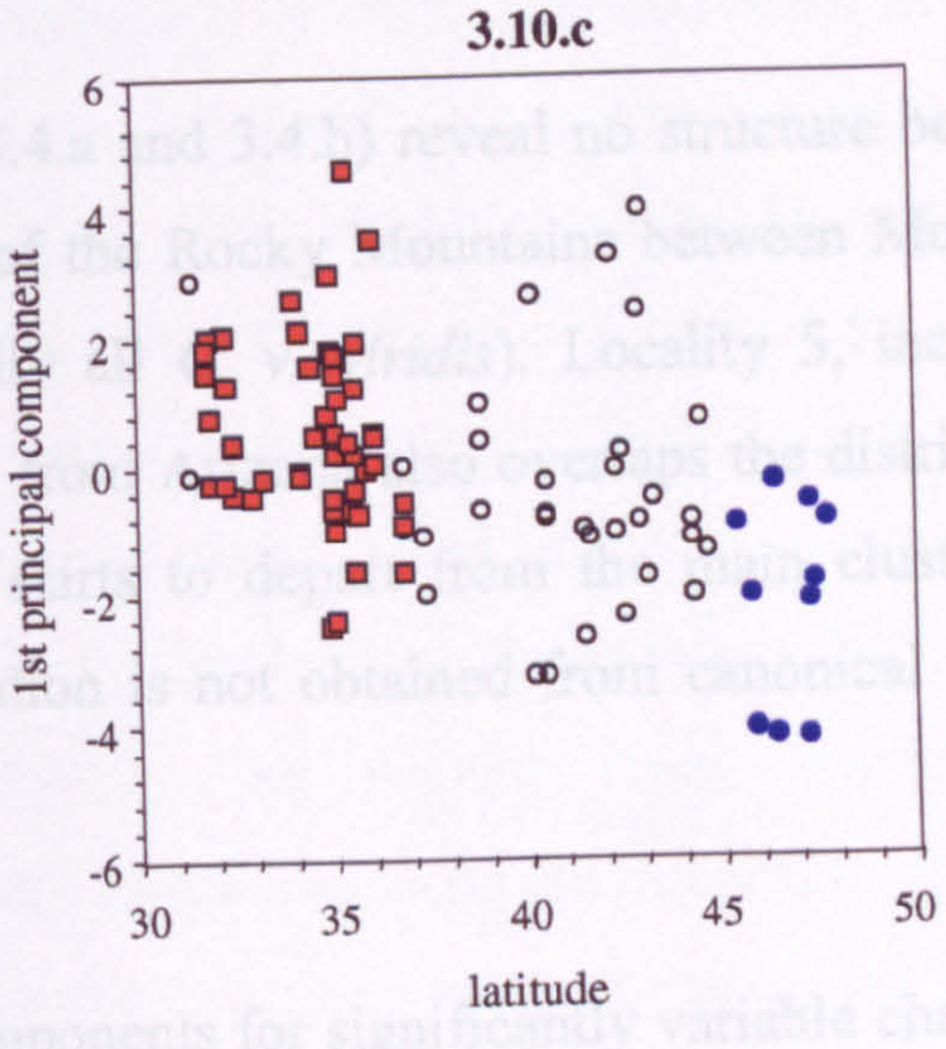
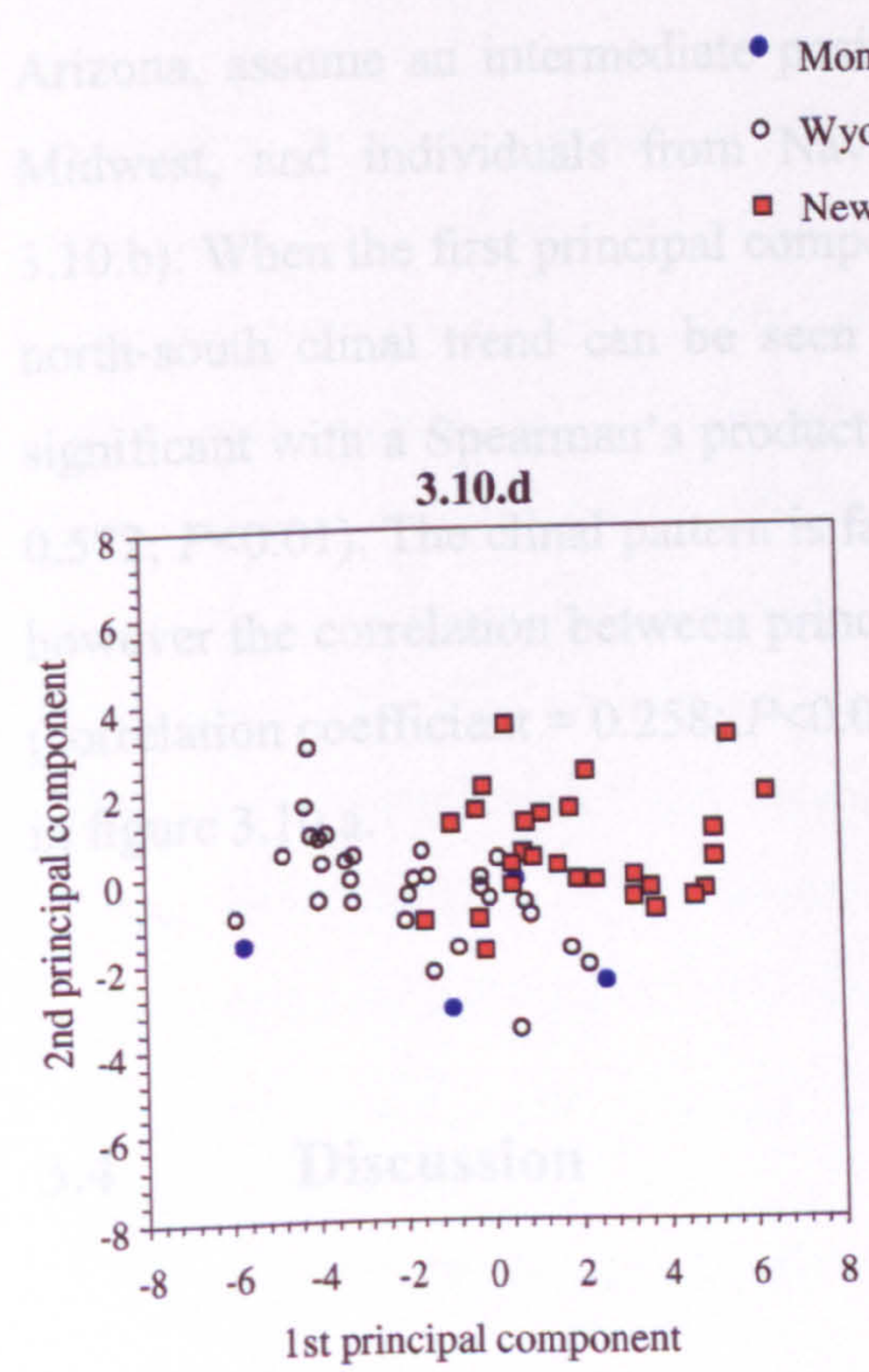


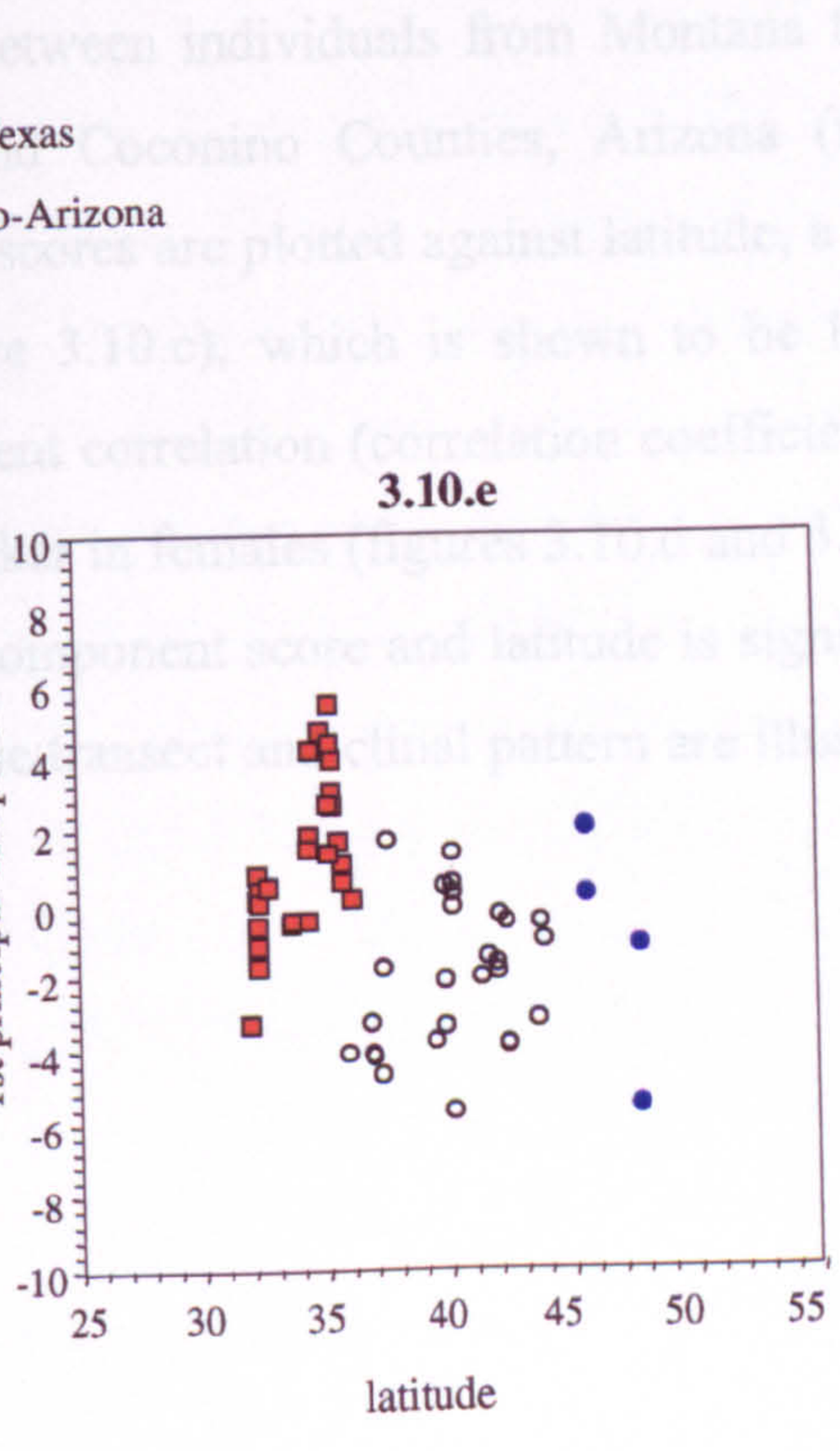
Figure 3.10. Weak clinal variation in *Crotalus viridis* from Montana, and south through Wyoming and Colorado, to Arizona, between the conventional subspecies *C. v. viridis* and *C. v. nuntius*: **a)** map illustrating the transect line and distribution of morphological characters; For males and females respectively, **b & d)** scatter plots of the first two principal component scores (see main text for the character sets used) **c & e)** first principal component scores against latitude. The clinal pattern appears to be much stronger in males than females.











Montana

Wyoming-Texas

New Mexico-Arizona

3.3.4.iii viridis-nuntius *transect*

The CVA plots of all localities (figures 3.4.a and 3.4.b) reveal no structure between localities 1-4 (representing the area east of the Rocky Mountains between Montana, south to New Mexico, and conventionally all *C. v. viridis*). Locality 5, including conventional *C. v. viridis* or *C. v. nuntius* from Arizona also overlaps the distribution of the points of localities 1-5, but also starts to depart from the main cluster. As mentioned previously, additional information is not obtained from canonical variate analysis of localities 1 to 5 alone.

Plotting the first and second principal components for significantly variable characters between all populations of males, individuals from New Mexico and Apache County, Arizona, assume an intermediate position between individuals from Montana to the Midwest, and individuals from Navajo and Coconino Counties, Arizona (figure 3.10.b). When the first principal component scores are plotted against latitude, a weak north-south clinal trend can be seen (figure 3.10.c), which is shown to be highly significant with a Spearman's product moment correlation (correlation coefficient = -0.572; $P<0.01$). The clinal pattern is far weaker in females (figures 3.10.d and 3.10.e), however the correlation between principal component score and latitude is significant (correlation coefficient = 0.258; $P<0.05$). The transect and clinal pattern are illustrated in figure 3.10.a.

3.4 Discussion

Only three investigators have closely examined the patterns of geographic variation in *Crotalus viridis*, of whom only Klauber's work has been published. Klauber did not use a multivariate approach, but presented tabulated data, descriptive statistics, and correlation or regression analyses (e.g., Klauber, 1941). The *C. viridis* subspecies

classification by Klauber (Klauber, 1930, 1949, 1956, 1972), is also entirely based on qualitative differences, as was the typical approach of traditional taxonomists during the mid twentieth century. More recently, a multivariate approach was used in the PhD thesis of Quinn (1987), who examined in more detail all the conventional subspecies of *C. viridis*, and in the Masters thesis of Schneider (1986), who examined the contact zone between *C. v. helleri* and *C. v. oreganus* in southern California (south of Monterey). In both studies, however, the analysis is based on using subspecies as OTUs defined *a priori*, a practice which inevitably leads to the OTUs coming out as distinct categories (e.g., Reichling, 1995). The present study is the first comprehensive, rigorous assessment of the morphological patterns within *Crotalus viridis* since 1987, and in which OTUs have been defined using numerical methods to assess within-locality homogeneity, not *a priori* categories.

Irrespective of which characters might be used to nominate subspecies, the present study does not reveal discrete subspecies categories. These results highlight the stark contrast between a multivariate statistical approach to assessing intraspecific relationships, compared to the traditional, qualitative and simplistic numerical diagnoses (e.g. Klauber, 1949, 1956, 1972). Lack of congruence between conventional subspecies and multivariate defined categories is not unusual. Similar conflicts have been noted for other widespread, continental polytypic species of reptiles, often leading to the conclusion that many subspecies categories are invalid (Benton, 1980; Thorpe, 1984; Barker, 1992; Dorcas, 1992; Wüster *et al.*, 1992a; Wüster and Thorpe, 1992; Taylor and Buschman, 1993; Reichling, 1995; Zamudio *et al.*, 1997).

3.4.1 Patterns of geographic variation in *Crotalus viridis*

The three larger regional groups, although not clearly defined, are consistent between males and females (e.g. figures 3.3 and 3.4), however the population affinities within each group are less obvious.

3.4.1.i *Pacific coast region*

The range of the western Pacific coastal populations (*C. v. oreganus*, *C. v. helleri*, *C. v. caliginis*), extends more than 2000 miles from southern British Columbia (Canada) to central Baja California (Mexico), including Isla Coronado Sur, Los Coronados (Stebbins, 1985). Previously, the Pacific coast rattlesnakes were considered as one single subspecies, *Crotalus viridis oreganus* (Amaral, 1929), but later split into the subspecies *C. v. oreganus*, *C. v. helleri*, and *C. v. caliginis* (Klauber, 1949), on the basis that continental populations in the north are morphologically distinct in colour and pattern from the those in the south, and the Isla Coronado Sur population because it has been separated from the mainland for a long time (although Klauber does not dispute that this population, more than likely, has arisen from the continental populations of southern California or Mexico). The only character Klauber could find to differentiate *C. v. caliginis* and *C. v. helleri* was adult size (Klauber, 1949).

The validity of the three Pacific coast subspecies categories is highly questionable in light of the distinct lack of differentiation shown in the CVA plots (figures 3.4 and 3.6) and the significant north south clinal pattern (figure 3.8). The clinal pattern (figure 3.8) implies a gradient of change throughout the region, between the northern and southern Pacific forms, rather than a more specifically defined zone of intergradation between *C. v. oreganus* and *C. v. helleri*, for example along the line (from north to southwest) Cape San Martin (Monterey County) to Shandon (San Luis Obispo County) to Lebec (Kern County) (Klauber, 1972) or slightly south of this line (Schneider, 1986). Schneider (1986) identified two main Californian clines, the first being a general north-south gradual change in width of the last tail band, with a more gradual change along the coast than inland (possibly stepped), and the second being a longitudinal cline through Schneider's proposed intergrade zone moving inland from the coast from the Cuyama valley to the San Joaquin valley. This latter trend could not be tested in the present study due to insufficient sample sizes.

3.4.1.ii *East of the Rocky Mountains*

Populations east of the Rocky Mountains are not easily distinguishable from each other (figures 3.3 and 3.4). *C. v. nuntius* was nominated a new subspecies of the prairie rattlesnake (Klauber, 1935), on grounds of smaller body size, and differences in body scale counts (e.g. ventral scales and dorsal scale rows, counts that are typically associated with smaller body size). *C. v. nuntius* was also noted to vary considerably in colour. Klauber (1935) did not dispute, however, that *C. v. nuntius* is clearly a smaller version of the nominate race, *C. v. viridis*, and also describes a gradual change over a broad area between the Arizona and New Mexico forms, moving northwards to Colorado. Irrespective of the subspecies status, therefore, Klauber's observations agree with the north-south clinal pattern apparent in figure 3.10, indicating a gradual change between the two races.

3.4.1.iii *The Great Basin*

The Great Basin rattlesnake, *C. v. lutosus*, is strikingly different from the other populations with respect to colour pattern, and differs in various scale counts (Klauber, 1930). Generally, however, morphology throughout the Great Basin seems to be highly uniform. The island population (locality 12) from Anahoe Island, Pyramid Lake (Washoe County, Nevada) appears to differ only in size from the mainland populations, and are generally in poor condition - many of the specimens examined had subcutaneous cysts, and/or spinal deformities. Specimens from the Grand Canyon (conventionally *C. v. abyssus*) appear to be closer morphologically to the Great Basin rattlesnakes (*C. v. lutosus*) than to any other populations (figure 3.3).

The zone of intergradation between *C. v. lutosus* and *C. v. oreganus* stretches from Adams County, Idaho, along the Snake River in Idaho and the eastern border of Oregon, through Oregon to the Cascade Mountains, and south along the crest of the Sierra Nevada Mountains (at the lower elevations). Specimens examined from Adams

and Washington Counties, Idaho, were clearly intermediate between the Great Basin and Pacific Coast morphology, being closer to the Great Basin forms. Moving further north towards the border between Idaho and the southeastern corner of Washington (Nez Perce County, Idaho), the morphology becomes more Pacific Coast-like. The results suggest clinal change along the Snake River (figure 3.9) between the Great Basin (*C. v. lutosus*) and Washington state (*C. v. oreganus*) forms. The inclusion of northern Californian populations helps to substantiate that the northerly Pacific coast populations are *C. v. oreganus* rather than *C. v. lutosus*, in that these individuals share similar principal component scores, thereby confirming the clinal pattern between the two extremes. It would be interesting to determine whether any intergradation between the Great Basin and Pacific coast forms also occurs in the northeastern corner of California. A second contact zone is reported between *C. v. lutosus* and *C. v. viridis* in eastern Idaho along the Salmon River (Klauber, 1972), however specimens were not examined from this area for the present study.

3.4.1.iv *Anomalous populations:* C. v. concolor

The subspecies *Crotalus confluentus decolor* (Klauber, 1930) coincided with a new species description, *Crotalus concolor* (Woodbury, 1929). The subspecific status was retained, however, and the nomenclature later modified to *Crotalus viridis concolor* (Klauber, 1936). The conflicting placement of *C. v. concolor* in the present analyses (figures 3.3-3.5) is not surprising, since considerable morphological variation is exhibited by these populations (see Klauber, 1930). Those populations in the southeastern corner of Utah (San Juan County) closely resemble conventional *C. v. nuntius* and *C. v. viridis* in pattern, and are believed to meet *C. v. viridis* in this area, where the populations supposedly intergrade. In fact the specimens examined here from San Juan County may actually be *C. v. viridis* rather than *C. v. concolor* (see Klauber, 1972), and like *C. v. nuntius*, C. *v. concolor* is generally smaller in size than other *C. viridis*. Animals from Sweetwater County, Wyoming, on the other hand, are far more similar to the Great Basin or Grand Canyon rattlesnakes with respect to

blotch shape, colour, and the tendency for the pattern to fade ontogenetically. Other forms (for example from the type locality in Garfield County, Utah) show only slight or no pattern (Klauber, 1930, 1935). A question arises whether *C. v. concolor* may in fact be a morphological non-entity.

Irrespective of the morphological resemblance to different regional forms, however, it is worth recalling here, that in the mitochondrial DNA phylogeny (Chapter 2), all haplotypes of *C. v. concolor* formed a clade. These haplotypes were from Sweetwater County, Wyoming, and central western Colorado. The clade also included, however, one conventional *C. v. abyssus* from the Grand Canyon. Total within-clade variation does not exceed 0.6%, suggesting a very uniform genetic structure within this population throughout its entire range, over 300 miles between Flaming Gorge Reservoir in Sweetwater County, Wyoming, to the Grand Canyon. Clearly these mtDNA data suggest the existence of a clearly defined lineage of *C. v. concolor*, even though it may be poorly defined morphologically.

Examination of specimens from a wider range of *C. v. concolor*, together with further DNA sequencing (for example DNA samples were not available from San Juan County, Utah), is clearly required before the complexities of the morphological patterns can be elucidated further. The racial affinities of conventional *C. v. concolor* are of particular interest since these populations secrete a high molecular weight neurotoxin, Concolor toxin (related to Mojave and Crotoxin) in their venom, which demonstrates high lethality in mouse experiments (Glenn and Straight, 1977). The relevance and importance of venom variation is examined and discussed in Chapter 5.

3.4.1.v *Anomalous populations:* C. v. cerberus

The position of *C. v. cerberus* with the Pacific coast forms (figures 3.3 and 3.6) is not unexpected. For many years this population, although geographically remote from the Pacific Coast, was included under *C. viridis oreganus,* before Klauber (1949) later

split the taxon into four separate subspecies (*helleri, caliginis, oreganus*, and *cerberus*). The tendency to associate *C. v. cerberus* with *C. v. helleri* relates to the close similarity in colour pattern of these populations, particularly the ontogenetic change towards melanism. There are in fact distinct differences between *C. v. helleri* and *C. v. cerberus* with respect to dorsal blotch pattern, pattern of melanism, and certain head scale counts.

Intergradation between *C. v. cerberus* and other *C. viridis* has never been reported. There may be overlap in range with *C. v. nuntius* around the southern limits of the Coconino Plateau, and just north of the San Francisco Mountains. Intergrades from this area have never been found, however, and it is possible that these populations may be separated altitudinally (Klauber, 1935, 1949, 1972). The most eastern distribution of *C. v. cerberus* is thought to occur in the Gila or Apache National Forests, in Catron and Grant Counties, New Mexico (a continuation of the National forests from Arizona), where it may contact the nominate form (Degenhardt *et al.*, 1996), although museum records or captive animals to support this suggestion are not known. It would be interesting to know exactly how far *C. v. cerberus* does extend into the habitat transition zone between woodland and grassland.

3.4.2 The general pattern

Crotalus viridis clearly forms a continuum from east of the Rocky Mountains to the Pacific coast, and north-south from southern Canada to northern Mexico. Excluding island populations, there are no impenetrable geographic barriers to *Crotalus viridis*, which passes through and around both the Rocky Mountains and the Sierra Nevada Mountains. This massive distribution may be divided loosely into three regional morphological groups corresponding to east and south of the Rocky Mountains, the Great Basin, and the Pacific Coast. Two forms, *C. v. cerberus* from the Central Highlands, Arizona, and the Pinaleño, Galiuro, and Santa Catalina Mountains in the south of Arizona, and *C. v. concolor* from eastern Utah and Sweetwater County, Wyoming, do not form discrete groups. Rather these populations form connections

between the larger clusters. Within and between the major groups there is evidence of clinal pattern. A strong north-south clinal trend exists between Pacific Coastal forms *C. v. oreganus*, *C. v. helleri* and *C. v. caliginis*, and similarly between *C. v. lutosus* (Great Basin) and *C. v. oreganus* (Washington State). A weaker north-south clinal trend is found between extreme northern *C. v. viridis* (Montana) and *C. v. nuntius* on the southern limits of the Colorado Plateau in the northeastern corner of Arizona.

The results closely corroborate Klauber's observations, that the connections between some populations are more tenuous than others (Klauber, 1949), for example the Great Basin forms have a much closer affinity with the Pacific Coastal forms that those east and south of the Rocky Mountains. Whether or not *C. v. cerberus* is fully disjunct from other populations of *C. viridis* remains uncertain, but to date no morphologically intermediate forms have been recorded.

Yet more detailed assessments could certainly be made of the clinal variation throughout *C. viridis* populations if larger samples were used for each locality, hence alleviating the need to pool samples. Similarly differences in results obtained for males and females may have been affected by generally lower sample sizes of females than males, rather than the affects of sexual dimorphism. Other factors influencing the measurement of specimens is shrinkage of specimens due to long-term storage in alcohol (Klauber, 1972; Schneider, 1986).

The results obtained provide a useful platform on which to hypothesise potential causes of the observed patterns. To some extent the broader patterns between regions appear to reflect phylogenetic pattern, while patterns within groups, and the position of the anomalous populations (*C. v. cerberus* and *C. v. concolor*) may be associated more with local ecological conditions rather than phylogeny (e.g. Klauber, 1956; Sweet, 1985; Schneider, 1986). Causal hypotheses for the patterns observed in this section are tested and discussed in Chapter 4 (Causes of morphological pattern).f

Chapter 4. Causes of Geographic Variation in Morphology

4.1 Introduction

The principle of causation states that every phenomenon has a cause (Peters, 1991). Having established the patterns of geographic variation for a particular set of characters, the natural progression is to hypothesise the underlying causes of the patterns. Explaining causes of geographic variation is central to the understanding of the speciation process, a typical example being the idea that allopatry (disjunction) is a fundamental requirement for speciation (Endler, 1977, and references therein). Scientific philosophy postulates several categories of causal theory, of which experimental scientific investigation generally concerns regularity theory. Causal regularity seeks simple correlation between cause and effect, implying that if cause is observed, so will be effect, and requires that any changes in the cause should also produce appropriate changes in the effect (Peters, 1991). The existence of a significant correlation does not confirm causation (Peters, 1991), but means that a causative hypothesis cannot be rejected. However, even if correlation does not mean causality, the absence of correlation is sufficient to warrant withdrawal of that causal hypothesis (Legendre and Fortin, 1989).

Patterns of geographic variation in genetically controlled morphological features may be explained by natural selection (ecogenetic factors; *sensu* Thorpe *et al.*, 1991) and historical causal factors (phylogenesis) (Thorpe, 1975a, 1979, 1987; Endler, 1982; Fleischer and Johnston, 1982; Malhotra and Thorpe, 1991a). Ecogenetic factors consist of the balance between gene flow and natural selection for current conditions, which may be biotic (e.g. mimicry, character displacement and interspecific competition), or physical (e.g. adaptation to environmental conditions such as substrate, climate, etc.). Phylogenesis refers to the divergence of lineages, that have become isolated following historical, geological, or climatic events (Endler, 1977; Thorpe, 1987; Thorpe *et al.*, 1994), such as the effects of genetic drift and selectively

neutral mutations (Thorpe *et al.*, 1994). Two well known examples of historical causal hypotheses include Pleistocene refuge theory, island colonisation and vicariance (Endler, 1982). In both instances, the patterns of variation can change if the population ranges shift later on, causing either further vicariance within the population, or secondary contact with the original founder population (Thorpe, 1987). Postglacial range expansion and contraction (refuge theory) has been proposed to explain the similarity in north-south orientation of transition zones in the grass snake, *Natrix natrix*, with those of other vertebrate species in central Europe (Thorpe, 1987). In North America, range shifts may also explain the pattern of geographic variation in the spotted frog complex, *Rana pretiosa*, in which levels of heterozygosity and allele number closely resemble the patterns observed in other species with similar distributions (Green *et al.*, 1996, and references therein). The above studies typify the benefit in comparing patterns of other organisms when exploring phylogenetic hypotheses.

There has been a recurring tendency for bias in interest toward one type of hypothesis, either ecology or phylogeny, while ignoring the other (Endler, 1982; Frumhoff and Reeve, 1994). In reality both main factors are likely to influence patterns of geographic variation, particularly morphological patterns (Thorpe, 1987; Thorpe, 1996). Furthermore, several factors contributing to a given pattern of variation are often strongly intercorrelated. The importance of both considerations is emphasised in studies where several causal hypotheses (ecogenetic and phylogenetic), were tested simultaneously using partial Mantel tests. For example in the Canary Island lizard *Gallotia galloti*, partial regression analysis of colour pattern variation with causal hypotheses revealed that blue leg spots appear to be closely associated with phylogeny while blue markings on the trunk or neck and yellow dorsal bars are strongly influenced by ecogenesis (Thorpe, 1996). Similarly, colour pattern in the Tenerife gecko, *Tarentola delalandii*, is thought to have arisen from ecological selection, while various body dimensions are associated with historical factors, and scalation characters are influenced by both phylogenetic and ecogenetic factors (Gübitz *et al.*, 2000).

Given that all populations are subjected to continuous selection pressure for maximal fitness (Mayr, 1970), it is perhaps not surprising to discover more examples where morphological variation is explained by ecological conditions rather than phylogenesis, including the skink genus *Chalcides* (Brown *et al.*, 1991), *Anolis* lizards (Malhotra and Thorpe, 1994), the green toad *Bufo viridis* (Castellano and Giacoma, 1998), the pitviper genus *Bothrops* (Wüster *et al.*, 1997), the Malayan pitviper *Calloselasma* (Daltry, 1995) and the salamander *Plethodon cinereus* (Lotter and Scott Jr., 1977). The profound and rapid evolutionary effects of directional selection on particular characters (or morphotypes) have been demonstrated very successfully in manipulative field experiments, where *Anolis* lizards were translocated to contrasting habitats (Malhotra and Thorpe, 1991b, 1994, 2000a; Thorpe and Malhotra, 1992; Losos *et al.*, 1997). The short-term (two-month) translocation study of *Anolis oculatus* (Malhotra and Thorpe, 1991b), revealed that the magnitude of morphological variation between survivors and non-survivors correlated with magnitude of ecological change experienced by the translocated individuals, and the season when translocation took place. A similar, long-term project involving translocation of *Anolis sagrei* to islands with different habitats, (Losos, 1997), recorded correlation between the magnitude of actual morphological differentiation over a 10-14 year period and the magnitude of vegetational variation.

Where ecogenetic variation prevails, individual characters can be subject to different selection pressures and are consequently vulnerable to being obscured by the generalised multivariate pattern (Thorpe *et al.*, 1994), as exemplified by the functional partitioning recurrently noted in colour pattern characters of various lizards. In *Gallotia galloti* the colour pattern of sexually mature males may be a balance between disruptive crypsis (yellow dorsal bars) and sexual selection (blue lateral markings involved in conspecific signalling) (Thorpe and Brown, 1989; Thorpe *et al.*, 1994; Thorpe and Malhotra, 1996). In *Anolis oculatus* general scalation is related to rainfall, while general body shape and colour are related to vegetation type. The reverse is true, however, for individual characters, for example the relative size of enlarged lateral scales, which is associated with vegetation, and the cyan element of body hues, which

is related to rainfall, not vegetation (Malhotra and Thorpe, 1991a; Thorpe and Malhotra, 1996).

Intraspecific variation may also result from phenotypic plasticity (non-genetic, environmental induction) (Dobzhansky, 1982; West-Eberhard, 1989) which might easily be confused with adaptation to local environmental conditions (Forsman and Shine, 1997; Malhotra and Thorpe, 2000b). Phenotypic plasticity is typically defined as 'the change in the expressed phenotype of a genotype as a function of the environment' (Bradshaw, 1965). Thus, plasticity can also defined as the ability of the genotype to produce more than one alternative form of morphology, physiological state, and/or behaviour in response to environmental conditions (Skúlason *et al.*, 1999). Phenotypic plasticity has been reported in fish (Skúlason *et al.*, 1999), frog tadpoles (Van Buskirk and Relyea, 1998), salamanders (Jockusch, 1997), lizards (Sorci *et al.*, 1996; Shine *et al.*, 1997b; Qualls and Shine, 1998; Losos, 2001) and snakes (Madsen and Shine, 1993; Queral-Regil, 1998). Temperature during embryonic development has been shown to have ontogenetic effects, for example influencing size in garter snakes (*Thamnophis sirtalis*) (Arnold and Peterson, 1989), and pine snakes (*Pituophis melanoleucus*) (Berger, 1989), scalation in garter snakes (*T. elegans*) (Fox, 1948; Fox *et al.*, 1961), and development rate in the green tree frog (*Hyla cinerea*) (Blouin *et al.*, 1998). The identification of plastic characters is not a straightforward procedure, but some reassurance may be gained from congruent patterns across many different categories of morphological characters.

4.1.1 Aim

In this chapter, five causal hypotheses of geographic variation in morphology of *Crotalus viridis* are tested using matrix correspondence (Mantel) tests (Mantel, 1967; Mantel and Valand, 1970; Sokal, 1979; Smouse *et al.*, 1986; Legendre and Fortin, 1989; Manly, 1991). One historical hypothesis, phylogeny as estimated from mitochondrial DNA, and four current hypotheses, including geographic proximity, vegetation, rainfall, and temperature, are tested. Hypotheses found to be statistically

significant for a given morphological pattern in pairwise tests are combined into simultaneous comparisons to investigate putative causes when the effects of intercorrelation between them are partialed out.

4.2 Methods

4.2.1 Definition of dependent variables

The characters used are as follows (all measurements are in millimetres unless stated otherwise, and all mensural data were adjusted by regression for size):

1 General morphology (**genmorph**): all individual characters included (listed and defined in chapter 3)

2 General head shape (**headshape**):
- head length (proximal tip of mandible quadrate to anterior edge of snout
- head width at widest point (gape)
- head width at eye level
- distance between eye and pit
- distance between pit and nostril

3 Head size (**headsize**) – includes head length, head width, and snout length
- head length (proximal tip of mandible quadrate to anterior edge of snout
- head width at widest point (gape)

4 General pattern (**genpat**):
- number of dorsal blotches
- number of tail blotches
- ventral mottling
- blotch length at margins and centre (mean of measurements for three blotches; units are number of dorsal scales) 25%, 50% and 75% VS positions along the body
- blotch width (units = dorsal scale rows)

5 General dorsal blotch shape at 25% along body (**genblot1**)
(units = dorsal scales):
- length of blotch margin
- length of midline of blotch
- blotch width

6 General dorsal blotch shape at midbody (**genblot2**) – as for no. 5

7 General dorsal blotch shape at 75% along body (**genblot3**) - as for no. 5

8 General dorsal blotch width (**genblotw**)
- number of dorsal scale rows, at 25%, 50% and 75% VS positions

9 Number of dorsal blotches (**dorsblot**)

10 Number of tail blotches (**tailblot**)

11 Ventral mottling (**ventmot**)

12 General body scutellation pattern (**bodyscut**)
- dorsal scale row counts at all positions (see no. 15)
- all tail scale row counts (see no. 16)
- number of ventral scales

13 General head scutellation (**headscut**):
- number of supralabials, infralabials, intersupraoculars, prefoveals, interoculabials (number of scale rows between the subocular and supralabial scales)v

14 Number of ventral scales (**ventrals**)

15 General pattern of numbers of dorsal scale rows along the body (dorsrows) – the numbers of dorsal scale rows counted at 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100% VS positions

16 General pattern of numbers of tail scale rows (**tailrows**)
- counted at 25%, 50%, and 75% CS positions

4.2.2 Hypothesis testing

Five hypotheses, one historical and four reflecting current processes, are proposed as possible causes of the present observed patterns of geographic variation in morphology in *Crotalus viridis*:

1) Morphology may reflect phylogeny.

Mitochondrial sequence information represents independent data that has the greatest chance of selective neutrality (Avise *et al.*, 1987; Avise, 1994; Thorpe, 1996). In this respect, the mitochondrial phylogeny is seen here to represent a neutral hypothesis, in the sense that phenotypes are selectively neutral and consequently "drift" through time. Therefore, it is predicted that morphological differentiation is proportional to patristic distance (the genetic distance along the branch lengths of a tree between two OTUs).

The historical hypothesis is tested using the mitochondrial cytochrome *b* (678 bp) and ND4 (652 bp) phylogeny presented in Chapter 2. The observed pattern of variation is predicted to correlate with the major clades of the *Crotalus viridis* phylogeny. In effect, the prediction being made is that dissimilarity is proportional to patristic distance. Matrices of dissimilarity coefficients representing phylogeny were constructed from patristic distances between OTUs, extracted from the maximum likelihood mitochondrial phylogeny in PAUP (e.g., Thorpe, 1996). Given that mitochondrial sequences have not been produced for all the individuals examined, haplotypes had to be assigned to each individual according to locality. Individuals were excluded from the analysis if either the mitochondrial haplotype for a particular locality was unknown (e.g. *C. v. concolor* from Utah), or the identification of individuals was uncertain (for example those from contact zones such as that in Idaho), or a locality was represented by more than one haplotype from different clades (e.g. *C. v. abyssus* in the Grand Canyon).

2) Variation is associated with geographical proximity, whereby morphological dissimilarity increases with distance (e.g., Wade and McCauley, 1988; Green *et al.*, 1996).

A fundamental aspect underlying hypotheses of geographic proximity is the influence of genetic exchange on diversity. Intuitively, greater opportunity for exchange of genes, encoding certain characteristics, is expected between neighbouring individuals, demes, or populations. Thus, spatially-close individuals are predicted to show a higher degree of morphological similarity than remote conspecifers (see Endler, 1977; Daltry *et al.*, 1996b; Green *et al.*, 1996). Neighbouring regions are also more likely to share common biotic and abiotic environmental conditions, causing convergent evolution in some characters among spatially close individuals, resulting from similar selective pressures (see Daltry, 1995).

Geographical proximity was calculated from latitude and longitude coordinates for each locality (see Appendix IV).

3) Variation is associated with habitat (vegetation) type.

Cryptic colour pattern in rattlesnakes and many other species of viper may be an adaptation for concealment against predators (Klauber, 1972; Kardong, 1980; Sweet, 1985; Greene, 1992; Forsman, 1995) and/or camouflage from prey since many are "sit and wait" (ambush) predators (Greene, 1992; Sazima, 1992; Diller and Wallace, 1996; Wüster *et al.*, 1997).

Vegetation type, or habitat was determined from a binary matrix, constructed for 28 vegetation categories where a score of 1 was placed against the appropriate category for each individual (e.g., Sokal, 1979). The vegetation types (Appendix VII) were identified from a map of potential natural vegetation (scale: 1:750,000; A. W. Küchler,

University of Kansas, 1966) from the National Atlas of the United States of America (United States Department of the Interior Geological Survey, 1970).

4) Morphological variation is related to temperature and

5) Morphological variation is related to rainfall

Patterns of scalation are documented to be influenced by temperature and/or rainfall in lizards (Soule and Kerfoot, 1972; Malhotra and Thorpe, 1997b) and snakes (Klauber, 1972; Osgood, 1978; Castellano *et al.*, 1994). In rattlesnakes, higher scale counts and keeling are noted for snakes from arid areas, and may be attributed to minimising water loss. Similarly snakes from arid areas are paler in colour, while the tendency towards melanism increases with altitude and in wetter environments (Klauber, 1972).

Data for the climatic variables, mean monthly temperature (°C), and mean monthly precipitation (millimetres) between and including the months of March to September, the annual activity period of western rattlesnakes, were extracted from maps (scale 1: 10,000,000) from The Climatic Atlas of North and Central America (WMO, UNESCO, 1979). Temperature (in 2.5°C increments) or rainfall (5 mm increments) are indicated on the appropriate climate maps by contour lines, with coloured zones between contours representing the range. Most localities fall within a range, rather than on the contour lines, hence the higher range values were recorded in each case.

Climatic conditions in particular vary little over very wide areas in the western US, especially over the more arid regions. In contrast, climate is noticeably more heterogeneous around the Pacific Coastal area. A hierarchical approach was therefore adopted, to test whether associations of morphological variation in the major geographical regions changed when either more, or less climatically heterogeneous

regions were excluded. A series of tests were performed, in each case treating males and females separately, starting with all individuals, and gradually reducing the analysis to focus on progressively smaller regions. Second in the sequence, all individuals west of the Rocky Mountains were tested (from the Great Basin, Pacific Coast, Arizona Central Highlands), followed by the Great Basin and Pacific Coast together, then the Pacific Coast independently, and finally individuals east and south of the Rocky Mountains.

Pairwise Mantel tests were used to identify significant relationships between each character and each independent putative cause. Partial Mantel tests were then used to test the pattern of variation in each character (dependent variable) against all significant causal hypotheses (independent variables) simultaneously, thus partialing out the effects of intercorrelation between the respective hypotheses. Causal hypotheses for which there was no significant pairwise correlation with a given character were excluded from the relevant simultaneous tests.

Geographic proximity was not examined in pairwise tests, since this factor had to be included in all simultaneous tests irrespective of a significant correlation with a given character, to regress out the effects of spatial autocorrelation arising from uneven distribution of sample sizes per locality.

Matrices and columns of dissimilarity coefficients were constructed using the programs DFmat350 and DFjoin350 respectively (by R. S. Thorpe). All Mantel tests were performed using B. F. J. Manly's program (modified by Thorpe) DFMant350, specifying 10,000 randomisations. The null hypothesis of no association was rejected if $P<0.05$. Given that the partial Mantel tests involved tests of multiple hypotheses, row-wise sequential Bonferroni corrections (Rice, 1989) were applied to all the probabilities obtained to correct for the number of hypotheses for each character.

4.3 Results

4.3.1 Pairwise Mantel tests

The results of all pairwise tests for each independent causal hypothesis are given in Appendix VIII. Tests of all individuals revealed significant pairwise relationships between most of the characters and all hypotheses, temperature being the least important hypothesis in both males and females. Progressively fewer significant relationships are found, not only as the number of individuals being compared is decreased, but also as more specifically defined regions are considered. Among individuals from the region east and south of the Rocky Mountains, scalation characters stand out as having the greater number of significant pairwise results. Body-pattern characters appear to be more important in individuals west of the Rocky Mountains, and most characters show significant relationships with patristic distance and vegetation. No obvious trend emerges from the tests of individuals from the Great Basin-Pacific Coast region, with the majority of characters having significant pairwise associations with several causal hypotheses. Among the Pacific Coast individuals alone, most significant associations are with body-pattern characters, and concern patristic distance and vegetation hypotheses.

4.3.2 Partial Mantel tests

As predicted, the number of characters significantly associated with each putative cause is reduced in the simultaneous tests, due to the partialing out of the intercorrelation effects between the hypotheses.

In tests of individuals across the range of *Crotalus viridis*, phylogeny stands out markedly as a cause of geographic variation in virtually all characters, in both males (n=236), Table 4.1.a, and females (n=175), Table 4.1.b. A highly significant association is found, in both sexes, between general morphology and causal

hypotheses of phylogeny and vegetation. In males, virtually all characters are found to be associated with phylogeny, and to a lesser extent, vegetation. Vegetation predominantly appears to explain variation in the body scutellation characters. Among the females, phylogeny together with geographic proximity (rather than phylogeny and vegetation, as is the case among the males), appear to explain variation in most characters. Neither temperature nor rainfall seem to be particularly important in either sex. Temperature appears to influence head size and shape, and to an extent blotch shape in males. Both sexes share a highly significant relationship between number of dorsal blotches and rainfall.

Phylogeny persists as the principal significant cause in all subsequent analyses west of the Rocky Mountains. Among male individuals (n=141) throughout the region west of the Rocky Mountains (Table 4.2.a), phylogeny explains mostly pattern, also general morphology and general scutellation. The same is true in females (n=112) (Table 4.2.b) although phylogeny also explains most of the scalation characters as well as pattern. Very few associations are found with remaining hypotheses, although in males, a significant association with vegetation is seen for general morphology and general pattern.

Phylogeny explains variation in virtually all characters in males (n=114), Table 4.3.a and females (n=102), Table 4.3.b, from the GB-PC region, with the exception of head-size and shape, ventral mottling, and dorsal or tail scale rows. No characters are significantly related to geographic proximity (except two characters in each case prior to Bonferroni correction). Rainfall is more significantly associated with pattern characters among individuals from the GB-PC region, explaining general pattern, and blotch shape, in both sexes. General morphology in the males is also associated with vegetation and temperature. Head size and shape also appear to be associated with temperature, while general pattern and blotch shape are significantly related to rainfall. In contrast, vegetation explains general scutellation and dorsal scale rows in the females, while temperature seems of little importance.

Results for the Pacific Coast region show a pronounced lack of significant relationships. For both sexes, several pattern characters are significantly associated with phylogeny. Among males (n=72), general morphology, general pattern, blotch shape at midbody and number of ventrals are significantly associated with vegetation. In females (n=70) general head scalation is also associated with phylogeny, general scutellation and ventrals with vegetation, and head shape with temperature. There are no other significant associations, and in general females tended to show far fewer significant pairwise relationships than males.

Among the eastern forms the number of informative associations is very few, and there is no clear pattern. In the males (n=95), the only relationships involve three scalation characters, which are all associated with rainfall including, general scalation, number of dorsal scale rows, and number of tail scale rows, although there is no clear pattern. Similarly, general scutellation and dorsal scale rows are also associated with rainfall in the females (n=63), together with vegetation. No trend is visible from the remaining three pattern characters which show significant relationships with entirely different hypotheses.

Table 4.1. Absolute standardised partial regression coefficients from Mantel tests of association between morphological characters/general morphological patterns in all male (**a:** n=236) and female (**b:** n=175) *Crotalus viridis* with five alternative causative hypotheses. ** indicates significant probabilities at the 1% level, * at the 5% level. Non significant results are given in reduced font size and shaded grey. Results significant before, but not after row-wise sequential Bonferroni correction are shaded grey, not reduced font size, and the significance level for these results indicated by asterisks. - indicates hypotheses that were not significant in the pairwise Mantel tests, and thus excluded from the simultaneous tests.

	Character	Phylogeny	Geographic proximity	Vegetation	Monthly temperature	Monthly rainfall
a)	genmorph	**0.2245****	-0.0031	**0.0595****	0.0671	0.0131
	headshape	**0.0752****	-0.0768	-	**0.1377****	-
	headsize	**0.0722****	-0.0482	-	**0.0888***	-
	genpat	**0.2956****	0.0320	0.0209	0.0539	0.0275
	genblot1	**0.1463****	-0.0272	0.0210	**0.1042****	0.0363
	genblot2	**0.1978****	-0.0263	**0.0469****	**0.0949****	0.0119
	genblot3	**0.1160****	0.1294*	0.0145	-	-0.0211
	genblotw	**0.1829****	**0.0939***	0.0213	-	-0.0755
	dorsblot	**0.1780****	0.0650	-0.0171	0.0276	**0.0131****
	tailblot	**0.3508****	0.0243	**-0.0359***	0.0562	0.0664
	ventmot	**0.4728****	0.0217	-0.0159*	0.0129	**-0.0539****
	genscut	**0.1695****	0.0139	**0.0488****	-	-0.0179
	genheadsc	**0.2246****	-0.0278	0.0341*	-	-0.0282
	ventrals	0.0093	-0.0298	**0.0399***	-	0.0693
	dorsrows	**0.0803****	-0.0549	**0.0344***	-	-
	tailrows	**0.1609****	-0.0363	**0.0327***	-	-
b)	genmorph	**0.0954****	0.1010*	**0.0697****	0.0180	0.0896
	headshape	-0.0238	**0.1498****	-0.0242	0.0732	0.0634
	headsize	-0.0077	**0.1806****	-0.0296	0.0883	0.0174
	genpat	**0.1071****	**0.1861****	0.0338	0.0028	0.0650
	genblot1	0.0214	0.0754	0.0061	0.0181	0.0758
	genblot2	**0.0459****	**0.1140****	0.0380	-	0.0024
	genblot3	**0.0788****	**0.2074****	**0.0503****	-0.0231	0.0708
	genblotw	**0.0482****	0.0493	-	-	-
	dorsblot	**0.0680****	**0.1116****	0.0407	**0.0778***	**0.1496****
	tailblot	**0.1185****	0.0940*	0.0428	0.0240	0.0515
	ventmot	-0.0238	**0.1498****	-0.0242	0.0732	0.0634
	genscut	**0.1150****	-0.0375	**0.0930****	-	-
	genheadsc	**0.1100****	-0.0373	-	-	-
	ventrals	-0.0112	**0.2085****	-0.0174	-	0.0300
	dorsrows	**0.0650****	**0.1363****	0.0214	-	**0.1607****
	tailrows	**0.1246****	**0.1314****	-	0.0376	-

Table 4.2. Absolute standardised partial regression coefficients from Mantel tests of association between morphological characters/general morphological patterns in male (**a:** n=141) and female (**b:** n=112) *Crotalus viridis*, west of the Rocky Mountains, with five alternative causative hypotheses. ** indicates significant probabilities at the 1% level, * at the 5% level. Non significant results are given in reduced font size and shaded grey. Results significant before, but not after row-wise sequential Bonferroni correction are shaded grey, not reduced font size, and the significance level for these results indicated by asterisks. - indicates hypotheses that were not significant in the pairwise Mantel tests, and thus excluded from the simultaneous tests.

	Character	Phylogeny	Geographic proximity	Vegetation	Monthly temperature	Monthly rainfall
a)	genmorph	**0.1451****	-0.0350	**0.0994****	**0.0999***	-0.0160
	headshape	-	-0.0905	-	**0.1611***	-
	headsize	-	-	-	-	-
	genpat	**0.2306****	0.0080	**0.0815****	0.0127	-0.0351
	genblot1	**0.1912****	-0.0602	0.0441	0.0108	-
	genblot2	**0.1248****	-0.0261	0.0560*	0.0558	-
	genblot3	**0.1596****	**0.1696****	**0.0524***	0.0451	0.0299
	genblotw	0.0477	0.1029*	0.0061	-0.0023	-
	dorsblot	**0.2038****	-0.0703	-	-	-
	tailblot	**0.1619****	-0.0469	0.0509	0.0274	-
	ventmot	-	-0.0334	0.0492*	-	-
	genscut	**0.0859***	0.0181	0.0266	-	-
	genheadsc	0.0593	-0.0066	0.0468*	0.0487	0.0301
	ventrals	-	0.0063	**0.0624***	-	-
	dorsrows	0.0506	-0.0006	0.0241	-	-
	tailrows	-	-	-	-	-
b)	genmorph	**0.1616****	0.0199	0.0385	0.0447	0.0465
	headshape	-	0.0829	-0.0019	0.1127	-
	headsize	-	**0.1187***	-	-	-
	genpat	**0.3208****	-0.0307	-0.0158	-	0.0556
	genblot1	**0.2430****	-0.0451	0.0290	-	0.0116
	genblot2	**0.2064****	0.0180	0.0030	-	-
	genblot3	**0.2997****	0.0288	0.0120	-0.0644	**0.1245***
	genblotw	**0.1303***	-0.0358	-	-	-
	dorsblot	**0.2601****	-0.0921	0.0118	0.0099	0.0779
	tailblot	**0.1954****	-0.0550	-0.0060	-	-
	ventmot	-	-	-	-	-
	genscut	**0.1383****	-0.0559	0.0281	-	-
	genheadsc	**0.1596****	-0.0836	-0.0181	0.0234	**0.1231***
	ventrals	**0.2030****	**-0.1114***	-	-	-
	dorsrows	-	-0.0299	**0.0697***	-	-
	tailrows	0.0716	-0.0717	-	-	-

Table 4.3. Absolute standardised partial regression coefficients from Mantel tests of association between morphological characters/general morphological patterns in male (**a:** n=114) and female (**b:** n=102) *Crotalus viridis*, from the Great Basin and Pacific Coast regions, with five alternative causative hypotheses. ** indicates significant probabilities at the 1% level, * at the 5% level. Non significant results are given in reduced font size and shaded grey. Results significant before, but not after row-wise sequential Bonferroni correction are shaded grey, not reduced font size, and the significance level for these results indicated by asterisks. - indicates hypotheses that were not significant in the pairwise Mantel tests, and thus excluded from the simultaneous tests.

	Character	Phylogeny	Geographic proximity	Vegetation	Monthly temperature	Monthly rainfall
a)	genmorph	**0.2621****	-0.1087	0.0690*	**0.1286***	0.0189
	headshape	0.0168	-0.0844	-	**0.1780****	-
	headsize	-	-0.0554	-	**0.1459***	-
	genpat	**0.3826****	-0.0831	0.0441	-0.0351	**0.1915***
	genblot1	**0.3210****	-0.0977	0.0074	0.0234	0.0981
	genblot2	**0.2162****	-0.0413	0.0490	0.0200	**0.1279***
	genblot3	**0.3291****	-0.0322	0.0200	-0.0681	**0.2016****
	genblotw	**0.1889****	-0.0028	0.0183	0.0173	0.0192
	dorsblot	**0.3745****	-0.1370*	0.0012	-0.0915*	0.2041
	tailblot	**0.3623****	**-0.1266***	-0.0497	0.0634	0.0429
	ventmot	-	-0.0213	**0.0805****	-	-
	genscut	**0.1235****	-0.0137	0.0401	-	-
	genheadsc	**0.1659****	-0.0955	0.0400	**0.0983***	0.0331
	ventrals	**0.1344****	-0.0599	0.0805	-	-
	dorsrows	0.0719*	-0.0165	0.0503	-	-
	tailrows	-	-	-	-	-
b)	genmorph	**0.2090****	-0.0466	0.0565	0.0575	0.0828
	headshape	-	0.0393	-0.0137	**0.1784***	-
	headsize	0.0280	0.0833	-	0.0711	-
	genpat	**0.4112****	-0.0807	-0.0563	-	**0.1268***
	genblot1	**0.3378****	-0.0917	-0.0806*	-	**0.1946***
	genblot2	**0.2743****	-0.0423	-0.0073	-	-
	genblot3	**0.3552****	-0.0457	-0.0116	0.0006	**0.1757****
	genblotw	**0.1659****	-0.0782	-	-	-
	dorsblot	**0.3299****	**-0.1551****	-0.0215	0.0429	**0.1416****
	tailblot	**0.2675****	-0.1079	-	**0.1372***	-
	ventmot	-	0.0507	0.0385	-	-
	genscut	**0.1129****	-0.1027	**0.1009***	-	-
	genheadsc	**0.1811****	-0.1020	-0.0042	0.0237	**0.1230***
	ventrals	**0.2486****	**-0.1746***	-	-	-
	dorsrows	-	-0.0841	**0.1113****	-	-
	tailrows	**0.1222****	-0.0573	-	-	-

Table 4.4. Absolute standardised partial regression coefficients from Mantel tests of association between morphological characters/general morphological patterns in male (**a:** n=72) and female (**b:** n=70) *Crotalus viridis*, from the Pacific Coast region, with five alternative causative hypotheses. ** indicates significant probabilities at the 1% level, * at the 5% level. Non significant results are given in reduced font size and shaded grey. Results significant before, but not after row-wise sequential Bonferroni correction are shaded grey, not reduced font size, and the significance level for these results indicated by asterisks. - indicates hypotheses that were not significant in the pairwise Mantel tests, and thus excluded from the simultaneous tests.

	Character	Phylogeny	Geographic proximity	Vegetation	Monthly temperature	Monthly rainfall
a)	genmorph	0.1108	-0.1184	**0.0969***	-	-
	headshape	-	-	-	-	-
	headsize	-	-	-	-	-
	genpat	0.0984	-0.0646	**0.1084****	-	-
	genblot1	0.0762	-0.0635	-	-	-
	genblot2	-	0.0187	**0.1072****	-	-
	genblot3	**0.1640****	0.0130	0.0111	0.0393	0.1427
	genblotw	**0.1834****	-0.0454	0.0006	-	-
	dorsblot	**0.1288***	-0.1192	0.0655	-	-
	tailblot	**0.1306***	-0.1436	-	-	-
	ventmot	-	-	-	-	-
	genscut	0.0471	-0.0517	0.0725	-	-
	genheadsc	0.0957	-0.0117	0.0386	-	-
	ventrals	-	-0.1495	**0.1290****	-	-
	dorsrows	-	-	-	-	-
	tailrows	-	-	-	-	-
b)	genmorph	0.0688	0.0249	0.0910	-	-
	headshape	-	0.0021	-	**0.2081****	-
	headsize	-	0.0782	-	0.1095	-
	genpat	**0.1142****	0.0419	-	-	-
	genblot1	0.0767*	0.4694	-	-	-
	genblot2	**0.0817***	-0.0158	-	-	-
	genblot3	**0.0855***	-0.0056	0.0772	-	0.1396
	genblotw	**0.1088****	0.0015	-	-	-
	dorsblot	-	-	-	-	-
	tailblot	-	-	-	-	-
	ventmot	-	-	-	-	-
	genscut	-	-0.1293	**0.1347****	-	-
	genheadsc	**0.0885****	0.0352	-	-	-
	ventrals	-	-	**0.1063***	-	-
	dorsrows	-	-	-	-	-
	tailrows	-	-	-	-	-

Table 4.5. Absolute standardised partial regression coefficients from Mantel tests of association between morphological characters/general morphological patterns in male (**a:** n=95) and female (**b:** n=63) *Crotalus viridis*, from East of the Rocky Mountains, with five alternative causative hypotheses. ** indicates significant probabilities at the 1% level, * at the 5% level. Non significant results are given in reduced font size and shaded grey. Results significant before, but not after row-wise sequential Bonferroni correction are shaded grey, not reduced font size, and the significance level for these results indicated by asterisks. - indicates hypotheses that were not significant in the pairwise Mantel tests, and thus excluded from the simultaneous tests.

Character	Phylogeny	Geographic proximity	Vegetation	Monthly temperature	Monthly rainfall
genmorph	0.2858	0.9981	0.3294	0.4810	0.1539
headshape	-	-	-	-	-
headsize	-	-	-	-	-
genpat	-	-	-	-	-
genblot1	-	-	-	-	-
genblot2	-	-	-	-	-
genblot3	-	-	-	-	-
genblotw	0.1954	0.4185	-	0.6326	-
dorsblot	-	-	-	-	-
tailblot	-	-	-	-	-
ventmot	-	0.1166	-	-	-
genscut	0.0698	0.0873	0.0142	-0.0885	**0.2533****
genheadsc	-	0.0495	0.0514	-	0.0674
ventrals	0.0991	-0.0110	-	-	-
dorsrows	0.1058	0.0651	-0.0219	-0.0529	**0.1951***
tailrows	0.0236	0.0568	-0.0326		**0.2582****
genmorph	-	0.1292	0.0123	-	0.1879
headshape	-	0.1137	-	-	0.1001
headsize	-	0.1689	-	-	0.0604
genpat	-	-	-	-	-
genblot1	-	-	-	-	-
genblot2	-	-	-	-	-
genblot3	-	-	-	-	-
genblotw	**0.0925****	-0.0108	-	-	-
dorsblot	-	0.0116	-	0.0477	**0.1848***
tailblot	-	-	-	-	-
ventmot	0.0678*	**0.1140***	-	-	-
genscut	-	-0.0209	**0.1542****	-	**0.1909***
genheadsc	-	0.0022	0.0787	-	-
ventrals	-	-0.0256	0.0830*	-	0.1367
dorsrows	-	0.0062	**0.1136****	-	**0.2322****
tailrows	0.0754*	-0.0052	0.0801	-	-

4.4 Discussion

In *Crotalus viridis* throughout the western U.S. the high association of most characters with phylogeny contrasts markedly with previous studies of other organisms. As mentioned in the introduction, morphological pattern in many reptiles and amphibians is more often explained by adaptation to current ecology than phylogenesis (e.g. Lotter, 1997; Brown, 1991; Malhotra, 1991a, 1991b, 1994; Losos 1997; Wüster *et al.*, 1997; Castellano, 1998; Gübitz *et al.*, 2000). The previous chapter showed that the number of significantly variable morphological characters decreases with the size of geographical region under consideration. Consequently fewer characters (individual or compound) are explained by causal hypotheses in progressively smaller regions. Phylogeny, however, remains the dominant explanation for patterns in morphology for the whole region, and sub-regions west of the Rocky Mountains. Further support for a phylogenetic cause of morphological differentiation in *C. viridis* stems from the close similarity in patterns of variation to the molecular phylogenetic pattern, as illustrated in the CVA plots (figures 3.3-3.4) in the preceding chapter. The main similarities are the correspondence of morphological groups and mitochondrial haplotype clades to the major geographic regions in the western United States, east of the Rocky Mountains, the Great Basin, and the Pacific Coast (see also Pook *et al.*, 2000).

There is pronounced sexual dimorphism in the second major causative explanation of morphological variation across the entire distribution. In females, there is strong correlation between most characters and geographic proximity. This is not the case among males. An interpretation of greater gene flow between spatially-close populations than more remote populations does not make sense unless geographic proximity is significant in both sexes. Geographic proximity, however, might contain other elements involved in ecogenetic forces acting on morphological characters (see Daltry, 1995). Neighbouring localities often have more similar local environmental conditions, such as climate, vegetation and fauna than distant ones. In this respect, it becomes difficult to speculate how such variables could directly act upon the evolution of a given character, although climatic factors and prey availability may influence

body size and certain morphological characters in the long-term (Klauber, 1972). For example, snakes living in hotter, more, arid parts of the species range, combined with a depauperate selection of prey species than in other regions, are predicted to have reduced scale counts and smaller body size, as happens to be the case in the dwarfed populations *C. v. nuntius* and *C. v. concolor* (Klauber, 1972).

Sampling regime might have influenced the difference in results between males and females, in that males were sampled from a greater number of localities (figure 3.2). Consequently the range of geographic distances between different localities in males differed from females, there being a higher representation of spatially-closer male individuals. This is likely to allow better discrimination between the effects of geographic distance and other intercorrelated hypotheses.

The strong association with vegetation in males (as oppose to geographic proximity) initially suggests stronger selection for crypsis in males. However, the characters significantly related to vegetation are mainly scalation characters, with only two, somewhat trivial pattern characters (dorsal blotches/bands in the lower third of the body and tail blotches) actually being significantly associated. Exactly how useful the vegetation categories used here are in reflecting habitat type may be questionable, since factors associated with vegetation may be more relevant than vegetation-type itself. In other words, specifically defined habitat types, or even better, the physical characteristics of different vegetation types (e.g. leaf size and shape, percentage canopy cover, vegetation density etc.), would probably make far more effective causative hypotheses. However defining habitat categories, or even generalising vegetation types, is not straightforward, rendering the process of category building somewhat subjective. Moreover, this task becomes practically impossible in studies relying on museum material with often imprecise locality information.

Irrespective of such considerations, both general morphology and general scutellation are significantly associated with vegetation in males and females, supporting the notion that crypsis against habitat is important for rattlesnakes in either providing

protection against predators (Klauber, 1972; Kardong, 1980; Sweet, 1985; Greene, 1992; Forsman, 1995) or concealment from prey (Greene, 1992; Sazima, 1992; Wüster *et al.*, 1997). Generalised pattern, however, is not correlated with vegetation in either sex, suggesting that the close intercorrelation of other characters with pattern might be important with respect to adaptation to particular habitats, rather than general pattern alone.

The results for all individuals do not support the theory of association between scalation and climate, where aridity is associated with higher scale counts (Klauber, 1972). No correlation was found between climatic causal hypotheses and either generalised or independent scalation characters, except dorsal scale rows among females. Temperature was concerned only with a few pattern characters (blotch shape in males and dorsal scale rows in females), and head size and shape in males.

The lack of association with climate is not totally surprising since some measurements, particularly rainfall, vary little over very wide areas in the western US, particularly in the more arid regions. Climatic pattern is most heterogeneous around the Pacific Coastal area, suggesting that the importance of climate might increase when this region is analysed independently. This prediction is not fulfilled, however, since no significant associations were found for males or females (except head shape with temperature in females). Five associations alone (2 among males, 3 among females) were revealed in the pairwise tests, but significance was lost in the simultaneous tests.

In stark contrast, rainfall (and no other hypothesis) appears to explain differentiation of scale characters among the eastern males. Morphological pattern is highly uniform among individuals throughout the Midwest to Arizona, but scale counts decrease moving southwards. The relationship of rainfall with scalation has been implicated as an adaptation to dehydration effects in lizards, for example in *Anolis oculatus*, in which an increase in body scale-number is associated with decreasing rainfall (Soule and Kerfoot, 1972), or vice versa in the lizard species *Chalcides sexlineatus* (Brown *et*

al., 1991). Similar observations have been made in a number of Californian snake species (including *Crotalus mitchelli*, but not *C. viridis*) across 10 genera, where the trend was an increase in number of scales moving towards more arid, desertified environments (Klauber, 1941). Klauber (1941) also noted sexual dimorphism for some species, including *Crotalus mitchelli*, in that males, but not females, followed the trend. Klauber excluded *Crotalus viridis* from the study on the grounds that this species does not occur in the more extreme desert conditions. An additional explanation for reduction in scale counts concerns reduction in body size. Klauber (1972) noted higher scale counts were correlated with larger body size. Body size might explain the greater tendency for scalation variation in males, since males grow much larger than females (Klauber, 1972; Diller and Wallace, 1996). Furthermore, it seems that the effects of climate and body size are additive, since the correlation between scale count and body size is particularly marked in animals from desert areas, but becomes less consistent in large animals from cooler environments. Scale counts do not change during the lifetime of the animal, however it is possible that scutellation may be influenced by environmental conditions, such as temperature, during embryonic development. Significantly decreased counts were noted in some scale characters of young *Thamnophis elegans*, born to wild caught females that had been maintained at cooler temperatures in captivity than their natural environment, throughout gestation. These findings suggest some (but not all) scale characters are phenotypically plastic (Fox *et al.*, 1961), and that not all scale characters are under the same genetic control. Note, however, that in these ovoviviparous snakes, gravid females are theoretically able to mitigate the effects of climatic extremes in nature, unlike in the laboratory. The conclusion that control of scalation is complex, and most likely polygenic, has also been drawn from captive breeding experiments selecting for particular scutellation and colour pattern aberrations in *Crotalus atrox* (Murphy *et al.*, 1987).

The general morphological uniformity in eastern *C. viridis*, particularly in pattern, between Montana and Arizona may explain the overall lack of correlations with any hypotheses. Given the evidence above, reduction in number of scales may be

associated with aridity and body size, and it is feasible that this trait is sexually dimorphic. Furthermore Klauber (1972) points out that trends in scalation with habitat are not always consistent intraspecifically (i.e. between subspecies). In contrast to the eastern forms, rainfall also appears to be an important factor with regard to pattern variation in the Great Basin and Pacific Coast individuals, including generalised pattern, and blotch shape characters. Colour pattern between the two regions is quite different. Pacific coast *C. viridis* have larger, rounder dorsal markings and generally darker overall coloration. Great Basin *C. viridis* have small, less pronounced, dark diamond-shaped blotches against a light sandy background. The association of pattern with rainfall might also reflect adaptation to reduce dehydration. Lighter coloration is often found in rattlesnakes from arid habitats, and may reflect heat to a certain extent, thus reducing water loss. A larger, darker colour pattern is often associated with moister, more vegetated habitats, where water loss is minimal. Dark coloration also aids heat absorption (Klauber, 1972), but also, darker colour is more likely to be cryptic in shadier environments with soil substrate.

The importance of colour pattern with respect to crypsis, however, cannot be disputed (Klauber, 1972), and the results highlight the potential for intercorrelation between adaptation for crypsis, and adaptation to different climatic conditions associated with contrasting habitats. A more realistic approach to testing crypsis as a causal hypothesis of colour pattern variation in rattlesnakes, would be to test for correlation between colour pattern type (e.g. speckled, large pattern, number of dorsal blotches, light or dark background, etc.) against the independent variables of microclimate, substrate colour, and substrate type (rock, gravelly, grass etc.). Such a test is more realistic, although in practise it is difficult when using faded museum specimens. Klauber describes the gradual change in *C. v. viridis*-*C. v. nuntius* coloration which closely follows the change in colour of substrate across the distribution. Snakes are greener in the grasslands of Montana and South Dakota, becoming yellow moving further south, reddish in the arid substrates of the Painted Desert in Arizona, then greener as the habitats become more lush again.

Some morphological variation in *Crotalus viridis* has been related to altitude (Klauber, 1972) but an altitudinal causal hypothesis was not tested here due to a deficit of altitudinal data for museum specimens and the general unavailability of specimens across a suitable altitudinal range in some localities.

In conclusion, phylogeny appears to be the dominant causal explanation for the observed patterns of morphological variation in *Crotalus viridis*. These results complement previous findings from the mitochondrial DNA study (Pook *et al.*, 2000); Chapter 2), and general patterns of morphological variation in the species (Chapter 3). Given the high influence of phylogeny, there were no parallels in patterns of variation between mitochondrial DNA variation and morphological characters as discovered in *Anolis* lizards by Malhotra and Thorpe (1994). The most important ecological influence across the region is likely to be vegetation (particularly in males), while other factors, including geographic proximity and climate seem superficial. The same is true for the Pacific region alone, whereas rainfall appears to be the more important factor for the Great Basin-Pacific Coast, and eastern Rocky Mountain regions.

The process of obtaining climatic and vegetational information for each individual is highly subjective and not necessarily straightforward. Information sources can vary considerably, particularly vegetation data. The present study used a highly detailed vegetation map, resulting in 28 vegetation categories, with the express purpose of providing as accurate a portrayal of habitat types as possible. In fact, simplifying the vegetation categories might have been more beneficial, since too many categories might obscure other ecological associations, in the sense that many vegetation types are actually very similar in terms of structure and thus, probable selection pressure on snakes. However, such categorisation cannot be carried out without using appropriate criteria. On the other hand, over simplification resulting in too few categories could also obscure the relationships (e.g., Schneider, 1986).

Results may be influenced by locality representation or distribution or uneven distribution of data points across a region, plausible explanations for the observed

differences between males and females. Alternatively, different associations between males and females may be due to different selective pressures associated with differences in behaviour in each sex. The independent hypotheses tested here might simply reflect other factors not taken into account explicitly, but most strongly correlated with one or other included independent variables.

This study has also shown how results change when progressively smaller regions are tested, for example comparing localised areas of extremely heterogeneous climate with expansive areas of uniform climate. To reiterate, the existence of a significant correlation does not confirm causation (Peters, 1991), but means that a causative hypothesis cannot be rejected. However, even if correlation does not mean causality, the absence of correlation is sufficient to warrant withdrawal of that causal hypothesis (Legendre and Fortin, 1989). Correlations between morphological characters and environmental gradients are often taken as evidence for natural selection. The possibility that these correlations are either coincidental, or environmentally induced, however, cannot be ruled out. Stronger evidence for natural selection comes from natural experiments of parallel patterns of geographic variation along similar environmental gradients between closely related allopatric species (Brown *et al.*, 1991; Thorpe and Malhotra, 1996; Skúlason *et al.*, 1999), as demonstrated for several species of lizards living on small islands, including the Canary Islands and the Lesser Antilles (Thorpe and Malhotra, 1996).

Widespread, continental, polytypic snake species, such as *Crotalus viridis*, present a far more challenging model for studies of causes of geographic variation in morphology, not only with respect to building appropriate data sets (e.g. substrate and habitat), but especially for comparing parallels, since comparative information on other species is profoundly lacking. The present study, therefore, serves as a platform upon which investigation of cause of morphological variation within *Crotalus viridis* can be developed and expanded in the future.

Chapter 5. Patterns and Causes of Venom Evolution

5.1 Introduction

5.1.1 Venom composition

Snake venoms are complex mixtures of proteins, nucleotides, inorganic ions, some carbohydrates and lipids, and other compounds, dissolved in approximately 80-90% water. Venom may be colourless, but yellows with increasing amounts of the enzyme, L-amino acid oxidase, an enzyme complex associated with riboflavin (Gold and Wingert, 1994; Meier and Stocker, 1995; Williams and White, 1997). Catalytic and non-catalytic protein or polypeptide components predominate. The nature and biological properties of snake venom components are peculiar to each species (Assakura *et al.*, 1992; Rodrigues *et al.*, 1998), giving rise to a formidable range of pharmacological effects, including serious local effects, such as severe tissue damage and necrosis, to potentially lethal systemic effects, such as circulatory shock, rhabdomyolysis, or respiratory paralysis (Russell, 1983; Meier and Stocker, 1995). Table 5.1 summarises the main categories of components and their main toxic effects (based on Meier, 1995). Comprehensive descriptions of venom composition, individual components, and pharmacology may be found in Russell (1983), Rosenberg (1990), and Meier (1995), and references therein.

5.1.2 Function of venom

There is no disputing the role of venom as a digestive juice. The venom gland is a form of salivary gland (Rage, 1994) that is associated with specialised morphology for feeding (including a highly kinetic jaw and skull structure, specialised teeth and musculature) and appropriate behavioural patterns (Pough and Groves, 1983; Kardong, 1996). Hence, it is widely accepted that the function of venom as a whole, relates to feeding rather than defence (Mackessy, 1993). The actual process of

dispatching prey and digestion in venomous snakes, however, involves a range of additional idiosyncrasies, determined by the mode of feeding employed by the predator. Rattlesnakes, for instance, are sit-and-wait (ambush) predators, and generally use a strike-release mechanism of envenomation (Furry *et al.*, 1991; Hayes and Duvall, 1991), relying on chemical cues to relocate prey once it has succumbed to the venom (Kardong, 1993; Lavin-Murcio *et al.*, 1993). Individual venom components are thus functionally partitioned, fulfilling a number of functions from the time of strike, to the point of ingestion. Some components are involved in immobilising prey (Minton and Weinstein, 1986; Pough and Groves, 1983), others commence digestion prior to the prey item reaching the stomach (e.g. low toxicity PLA_2s), and others are concerned with rapid killing (e.g. high toxicity PLA_2s) (Thomas and Pough, 1979; Kochva *et al.*, 1983). In contrast to rapid killing, it may be beneficial to delay time to death. Prey is prevented from escaping, but at the same time the digestive process is initiated via the blood circulation, allowing rapid spread of toxins to all the body tissues (Hayes, 1991; Mackessy, 1988; Mackessy, 1993; Faiz *et al.*, 1996). Such a toxic concoction also affords some protection from injury during feeding, and other components may serve less direct functions, such as neutralising bacterial toxins of the ingested prey to prevent putrefaction (Thomas and Pough, 1979; Kochva *et al.*, 1983; Pough *et al.*, 2001).

Table 5.1. A generalised summary of the main categories of snake venom components and associated toxicity. Additional notes are given where necessary. The actual range of pharmacological effects is detailed and physiologically complex. Different combinations of effects are specific to certain taxonomic groups, e.g. viperid venoms, crotalid venoms, *Bungarus*, *Dendroaspis*, African Elapids, etc.

LOCAL EFFECTS (proteins)		
Enzymes	- endopeptidases (e.g. serine proteases cysteine proteases, metalloproteinases) - exopeptidases	Digestive function leading to tissue breakdown. Many types (over 30) of which 10 common to all snake venoms.
Myotoxins	- small (5kD) basic non-enzymatic proteins - large (12-16 kD) basic phospholipase A_2s (PLA_2)	Myotoxic; cause myonecrosis; PLA_2s also cause various systemic effects; some have enzymatic activity. Particularly common in viperid & crotalid venoms.
Haemorrhagic toxins	- low and high molecular weights	All have proteolytic function; induce haemorrhage by diapedesis (erythrocytes leave intact endothelium through intercellular junctions) or per rhexis (endothelial cell lysed); many are fibrinogenases, preventing clotting.
SYSTEMIC EFFECTS (proteins)		
Neurotoxins	- postsynaptic - presynaptic (PLA_2s, e.g. Crotoxin & Mojave toxin, dendrotoxins, fasciculins)	Generalised rhabdomyolysis; muscle paralysis; potentially fatal if respiratory paralysis occurs. Basic phospholipase A_2 may be complexed with acidic, basic or neutral proteins
Cardiotoxic components	- Cardiotoxins - Sarafotoxins	toxicity associated with direct effects on the heart
Cardiovascular components	A range of toxins, target blood pressure, blood vessel walls, endothelial cells, blood platelets, or various stages of clotting	A range of components with activating or inactivating effects on nearly all phases of human haemostasis. Causes circulatory shock which can be fatal.
MISCELLANEOUS COMPONENTS (proteins/glycoproteins)		
Lectins		Important in cell membrane to cell membrane interactions
Nerve growth factors		Strong mast cell degranulating agents - may contribute to toxic effects
PLA2 inhibitors		Stablise PLA_2s
Components affecting the complement system		Lead to increased permeability to toxic components to enter the bloodstream
NON-PROTEIN COMPONENTS		
Amino acids		Origin uncertain. Do not contribute to toxicity
Biogenic amines (acetylcholine and other cholamines)		May contribute to causing pain at bite site
Carbohydrates (glucose, galactose, mannose; mucopolysaccharides)		May help protect of lumen of venom gland
Lipids (e.g. capric, lauric, linoleic, oleic, stearic acids)		Increase toxicity, e.g. in some PLA_2s
Nucleosides & nucleotides		
Riboflavin		Prosthetic group of L-amino acid oxidase
Organic acids (triglycerides, phospholipids, citrate)		
Anions (phosphate, sulphate, chloride)		
Cations (e.g. Na, K, Zn, Mg, Mn, Ca, Fe, Co, Ni)		Zinc may activate or deactivate some enzymes

5.1.3 Variation in venom composition

Intraspecific variation was presented in the General Introduction, and will not be repeated here. At this point, however, other forms of venom variation are introduced, aside from differences within species.

5.1.3.i *Ontogenetic variation*

Examples of ontogenetic variation in venom components and concentrations of components are numerous. In *Crotalus d. durissus* the relative concentrations of crotoxin, haemorrhagic and proteolytic components change with age (Gutiérrez *et al.*, 1991). Mackessy (1996) reports that the major metalloprotease, CVO protease V, occurs in adult venoms, but not in the neonate and juvenile venoms of *C. v. oreganus*. In general, proteolytic activity may increase with body size (Minton and Weinstein, 1986; Mackessy, 1988; Gutiérrez *et al.*, 1991), while PLA_2 activity tends to decrease, as for example in Pacific Coast *C. viridis* (Mackessy, 1988). L-amino acid oxidase is usually found in greater quantities in venoms of larger snakes, for example in *Bothrops*, *Agkistrodon*, and *C. viridis* (Jiménez-Porras, 1964; Bonilla and Horner, 1969; Fiero *et al.*, 1972). Ontogenetic variation is also noted in *Crotalus atrox* (Minton and Weinstein, 1986) and *Pseudonaja* (Williams and White, 1992).

5.1.3.ii *Sexual variation*

Sexual variation has been reported in venom yield, but not protein composition or toxicity, between male and female *Crotalus viridis* (Glenn and Straight, 1977). Similarly, sexual variation in venom composition is not apparent in *Echis carinatus* (Táborská, 1971). Two studies report extra protein bands in female *Bitis nasicornis* (Marsh and Glatson, 1974) and *C. adamanteus* (Mebs and Kornalik, 1984), although additional data would be required to support these findings.

5.1.3.iii *Seasonal variation*

Seasonal variation was noted in the venom of *Vipera ammodytes* (Gubenšek *et al.*, 1974), however, overall evidence of seasonal variation in venom composition is lacking (Mebs and Kornalik, 1984). Gregory-Dwyer (1986) failed to find any variation in isoelectrically focused protein profiles of captive *Crotalus viridis*, *C. atrox*, or *C. molossus*, that had been subjected to environmental temperatures and photoperiods comparable to those experienced by wild populations. Seasonal variation was noted in one individual of *Pseudonaja textilis* (Williams and White, 1992) with respect to enzyme activity and reactivity with antivenom.

5.1.4 Important venom components and venom variation in *Crotalus*

The myotoxin group of components are of particular interest and importance with respect to clinical pharmacology. Myotoxins in rattlesnakes contribute up to 30% of total venom protein (Aird, 1985). Following envenomation by rattlesnakes, myotoxins are responsible for the more serious local pathophysiological effects, including oedema, haemorrhage and myonecrosis (Ownby *et al.*, 1997). Moreover, *Crotalus* myotoxins provide excellent subjects for investigating the evolutionary origins of venom components. A range of myotoxins are represented in the venoms of different *Crotalus* species, and considerable intraspecific variation is evident, especially in the ability to secrete different homologues of myotoxic phospolipases A_2 (PLA_2) (Aird and Kaiser, 1985; Meier and Stocker, 1995; Ownby *et al.*, 1997).

The first major category of myotoxins includes non catalytic, low molecular weight (5 kD), basic polypeptides, for example crotamine and myotoxin *a* (Ownby *et al.*, 1988; Nedelkov and Bieber, 1997; Norris *et al.*, 1997). To date, these toxins have only been isolated from *Crotalus* venoms, including *C. adamanteus* and *C. durissus*, and at least six forms (including isomeric forms, e.g., O'Keefe *et al.*, 1996; Nedelkov *et al.*, 1997)

have been characterised from venoms of different populations of *Crotalus viridis*. The second group, PLA_2-myotoxins, includes large basic proteins (12-16 kD) with the primary sequence of a PLA_2, which may or may not have catalytic activity (Meier and Stocker, 1995; Ownby *et al.*, 1997). Despite all being very similar in primary structure, the larger toxins exhibit great diversity in pharmacological properties (Rosenberg, 1990). This has led to the suggestion that, although present in the venom of snakes from virtually every family and genus examined (Rosenberg, 1990), viperid and crotalid PLA_2s may have a different precursor to those of elapids or hydrophiid snakes, and represents an example of convergent evolution. Evidence to support this notion stems from the marked structural differences between elapid and hydrophiid Class I PLA_2s, and the PLA_2s in crotalids and viperids which conform to Class II toxins (Dufton and Hider, 1983; Harris, 1997). Among the elapid and hydrophiid Class I PLA_2s, three groups are recognised: i. Australian elapids and sea-snakes; ii. African and Asian elapids; iii. kraits, suggesting common ancestry among these snakes (Slowinski, 1997).

Well known examples of *Crotalus* PLA_2-myotoxins include the presynaptic neurotoxins, crotoxin (Aird and Kaiser, 1985 and references therein), and Mojave toxin (Glenn and Straight, 1978). Venoms that are positive for these toxins show markedly higher lethality. The intraperitoneal LD_{50} values for mice injected with Mojave-toxin positive *C. scutulatus* venom were 0.24 mg/kg, 11-fold higher than the LD_{50} values obtained with Mojave-toxin negative venom (2.80 mg/kg) (Glenn and Straight, 1978). A number of homologues of these molecules have also been isolated and characterised, including Concolor toxin (Pool and Bieber, 1981; Glenn and Straight, 1977), vegrandis toxin (Kaiser and Aird, 1987), and related toxins from *C. mitchellii* (Glenn and Straight, 1985b), *C. basiliscus, C. horridus* (Glenn *et al.*, 1983; Glenn and Straight, 1985a), and *C. lepidus* (Rael *et al.*, 1992). All these neurotoxic PLA_2s exhibit catalytic activity.

Other large, enzymatic, but non-neurotoxic myotoxin-PLA_2s are known from the venoms of several *Bothrops* species (Kaiser *et al.*, 1990; Gutiérrez *et al.*, 1984; Ménez,

1991; Rodrigues *et al.*, 1998). These PLA_2s do not appear to form part of the group of crotoxin-type neurotoxins (Ménez, 1991). More recently, a PLA_2-myotoxin has been characterised from *C. viridis viridis* venom, which has high homology to one isoform of the B component of crotoxin and, to a lesser degree, Mojave toxin, although this component is non-neurotoxic (Ownby *et al.*, 1997).

The most thoroughly investigated example of intraspecific variation in PLA_2 neurotoxins is that of *C. s. scutulatus*, the Mojave rattlesnake (Glenn and Straight, 1978; Glenn *et al.*, 1983; Rael *et al.*, 1984; Glenn and Straight, 1989; Wilkinson *et al.*, 1991). The venom secreted throughout most of the range of *C. s. scutulatus* (type A venom) is characterised by the presence of a high lethality PLA_2 (Mojave toxin) and a lack of haemorrhagic and proteolytic effects. Populations in central Arizona, however, do not secrete Mojave toxin, and this venom (type B) demonstrates marked haemorrhagic and proteolytic activity, and low lethality. Furthermore, hybrid A+B venoms have been identified, displaying various combinations of neurotoxic, haemorrhagic and proteolytic properties.

Crotalus viridis concolor (the midget-faded rattlesnake) is the only population of western rattlesnake to secrete a neurotoxic PLA_2 complex (Concolor toxin) in adulthood. The lethality of adult *C. v. concolor* venom (LD_{50} range 0.13-0.45) approximates the i.p.- LD_{50} values for type A *C. scutulatus* and *C. durissus terrificus* venoms, and is 10 to 30 times more lethal than all the other *C. viridis* subspecies (Glenn and Straight, 1977). Consequently, Concolor toxin is believed to be a homologue of the Mojave toxin or crotoxin in *C. scutulatus* and *C. durissus terrificus* venoms respectively. Very occasional reports exist of a similar component from *C. v. viridis* venom (Glenn and Straight, 1990). These findings are believed to be the result of either incidental hybridisation between *C. v. viridis* and *C. v. scutulatus*, or from interbreeding in the zone where *C. v. viridis* meets *C. v. concolor* (Murphy and Crabtree, 1985; Glenn and Straight, 1990).

5.1.5 Snakebite in the United States

In the United States, rattlesnakes account for approximately 65% of venomous snake bites, the majority of which result from irresponsible animal handling (Gold and Wingert, 1994; Gomez and Dart, 1995). The first symptoms of a rattlesnake bite include swelling, erythema or ecchymosis. Moderate envenomation also involves systemic signs and symptoms, including nausea, vomiting, oral paresthesias or unusual tastes, mild hypotension, mild tachycardia, and tachypnea. The systemic symptoms are much stronger in severe envenomation, which also include alteration of mental status, possibly other forms of respiratory compromise, and blood pathogenesis, such as abnormal coagulation parameters, serious bleeding or threat of spontaneous bleeding, and very low or undetectable counts of various clotting factors (Gomez and Dart, 1995). The most serious effect of envenomation by rattlesnakes is the severe local tissue damage caused by myotoxins (Ménez, 1991), and other effects leading to fatality, such as circulatory shock, severe haemorrhaging, and renal failure (Fan and Cardoso, 1995; Gomez and Dart, 1995; Gutiérrez, 1995). Additionally, rhabdomyolysis, and respiratory paralysis resulting from the presence of powerful neurotoxins, such as Crotoxin or Mojave toxin, greatly increase the chance of fatality (Gutiérrez *et al.*, 1991; Jansen *et al.*, 1992; Amaral *et al.*, 1997). Fatalities are usually a consequence of failure to administer, or inappropriate dosing of, antivenom (Gold and Wingert, 1994). *Crotalus viridis* is among the top five of most frequent offenders in the U.S.A. Snakebite in the U.S. is not as serious a problem as in developing countries, such as Asia or Africa, where incidence of snakebite is higher due to agricultural activity (Gold and Wingert, 1994). Out of 8,000 venomous snake bites in the U.S. per annum, about 9-14 are fatal, a stark contrast to figures for the rest of the world, where fatalities are estimated at 125,000 per annum (Chippaux, 1998). The main importance of ongoing venom research in the U.S., concerns the medical implications of the confusion in pharmacology arising from intraspecific venom variation. All bites are potentially fatal if administration of antivenom is delayed, especially in cases where symptoms are obscure, with very little pain or local tissue

necrosis, but pronounced neurotoxic symptoms, as is typical following type A *C. scutulatus* envenomation (Aird *et al.*, 1989).

5.1.6 Causes of venom variation

The preceding chapter introduced ecogenesis and phylogenesis as categories of causal hypotheses to explain patterns of geographic variation in genetically controlled, morphological features (Thorpe, 1975a, 1979; Thorpe *et al.*, 1991; Endler, 1982; Malhotra and Thorpe, 1991a). Variation may be also caused by environmental induction, which is not under genetic control (Bradshaw, 1965; West-Eberhard, 1989). As is the case with morphological characters, several intercorrelated causal factors are likely to be involved in shaping venom composition, since independent venom components appear to have different functions (Kardong, 1996). Genetic control, rather than phenotypic plasticity, was suggested to be explain consistent patterns in the presence or absence of certain proteins among individuals from a single litter of *Crotalus adamanteus* (Mebs and Kornalik, 1984). Variation in electrophoretic profiles and physiological effects of venoms from *Echis carinatus* (Táborská, 1971), and in the fer-de-lance, *Bothrops atrox* (Meier, 1986) were also attributed to genetically controlled factors. These studies did not test which forces might actually be responsible for causing the observed variation. However, Mantel tests (Mantel and Valand, 1970) were used to show that natural selection for diet might explain venom variation in *Calloselasma rhodostoma* (Daltry *et al.*, 1996a, 1996b). The historical hypothesis, that the degree of venom variation may be related to time of isolation, has been applied to populations of *Notechis scutatus* and *N. ater* on small islands (Williams *et al.*, 1988). A phylogenetic interpretation was also made from variation in electrophoresed protein profiles among populations of *Crotalus viridis* (Foote and MacMahon, 1977). Reproductive isolation followed by divergence is hypothesised to explain geographic variation in the venoms of *Bothrops nummifera* (now *Atropoides nummifer*), after populations became restricted to either the Atlantic coast or the Pacific coast by mountain barriers (Jiménez-Porras, 1964). Climate or diet were not

thought to have influenced venom variation. However, there are ecotypic differences each side of the mountains, suggesting a more rigorous, statistical causal investigation would be appropriate .

5.1.7 Aim

The aim in this chapter is to investigate patterns of intraspecific venom variation in *Crotalus viridis* from presence of absence or proteins, as revealed by isoelectric focusing. Three causal hypotheses of the resulting patterns of variation are tested, phylogeny, geographic proximity, and biotope, using partial Mantel tests. The origin of the high lethality Concolor toxin in the venom of just one population of *C. viridis* (*C. v. concolor*) is also considered.

5.2 Methods

Venom samples were extracted by encouraging hand-held snakes to bite voluntarily through an artificial membrane stretched over a collecting vessel. The venom glands were not massaged, thus ensuring clean fresh samples with a minimum of cellular debris. Samples were taken from 56 individuals of *Crotalus viridis* from across the species range (figure 5.1). Some individuals were milked twice, but on separate occasions, bringing the total number of samples to 67. All venoms were desiccated within 12 hours of collection, by leaving the sample tubes open-topped to stand in silica gel. When ready to use, the samples were re-dissolved in ultrapure water and isoelectrically focused (IEF) across polyacrylamide gels containing carrier ampholytes.

Isoelectric focusing (IEF) is a straightforward electrophoretic method, widely used for qualitatively comparing the composition of small venom samples (e.g., Tu and Adams,

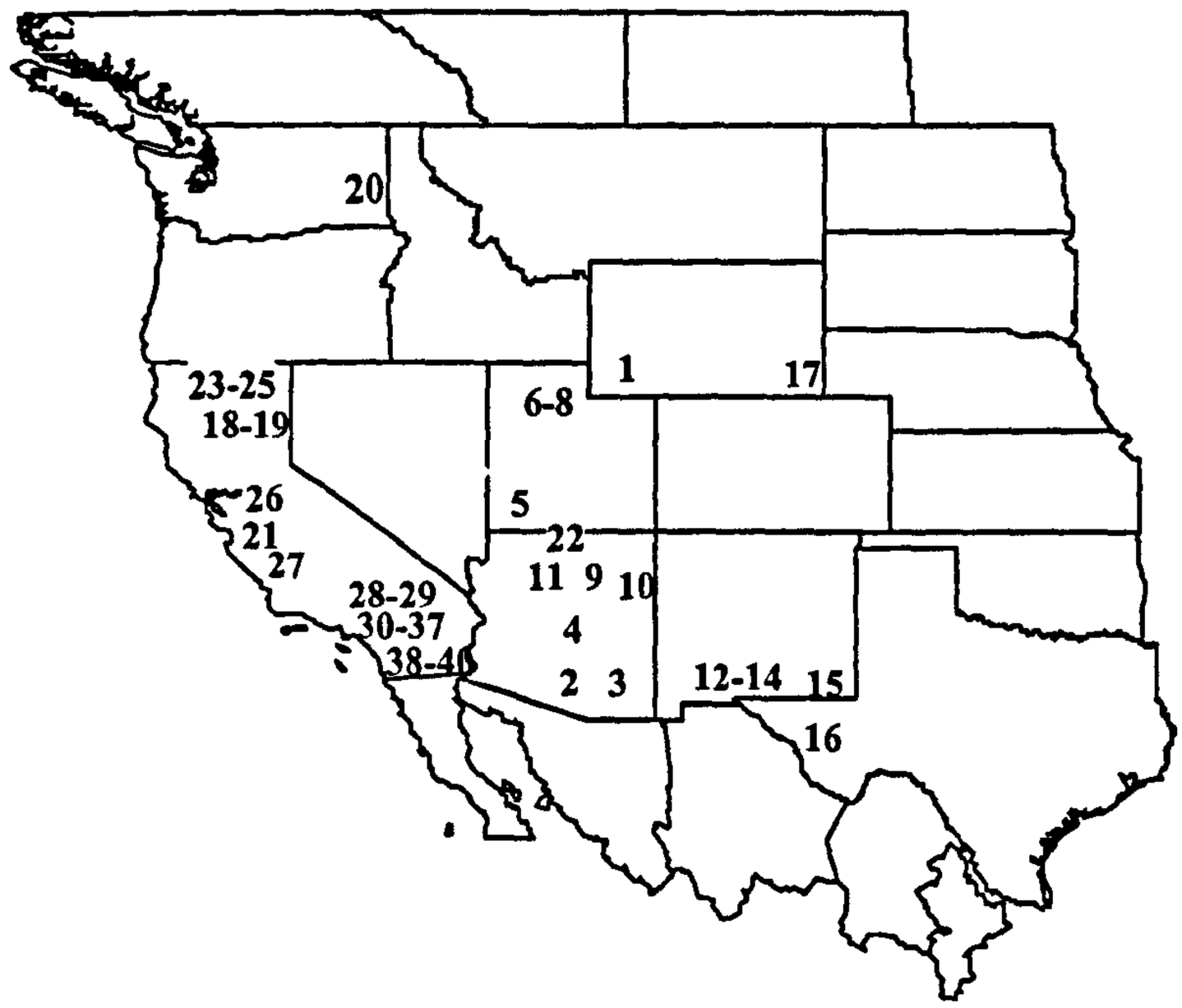


Figure 5.1. Distribution of unique IEF protein profiles for the venom of *Crotalus viridis*. Refer to Appendix IX for precise locality details.

1968; Bonilla and Horner, 1969; Táborská, 1971; Jones, 1976; Young *et al.*, 1980; Chippaux *et al.*, 1982). Proteins, or other amphoteric molecules, are separated according to net charge through an artificial pH gradient. A stable pH gradient increasing progressively from anode and cathode is established by electrolysis of carrier ampholytes in a suitable anticonvective liquid medium. When introduced into the system, a protein or other amphoteric molecule will migrate according to its surface charge in the electric field. Should the initial charge be positive, the molecule will migrate towards the cathode to regions of higher pH, while gradually losing positive charges and gaining negative charges. Eventually, the molecule reaches a zone where its net electrical charge is zero, which is the isoelectric point (pI) of the molecule. In maintaining an appropriate electric field, the protein is prevented from back diffusion. Thus an equilibrium is reached whereby the protein or other amphoteric macromolecule will become concentrated into a sharp band. The shallower the pH gradient, the better the separation (Righetti and Drysdale, 1976).

Sample details are provided in Appendix IX, and full laboratory protocols given in Table X.1, Appendix X. Gels were scored by eye for the presence (1) or absence (0) of protein bands. The isoelectric point (pI) for each band was calculated from the linear regression (pI = -0.7635x + 10) of the distance travelled from the cathode against a series of known isoelectric points of calibration marker proteins, electrophoresed alongside the venom proteins (figure 5.2; see Table X.2, Appendix X).

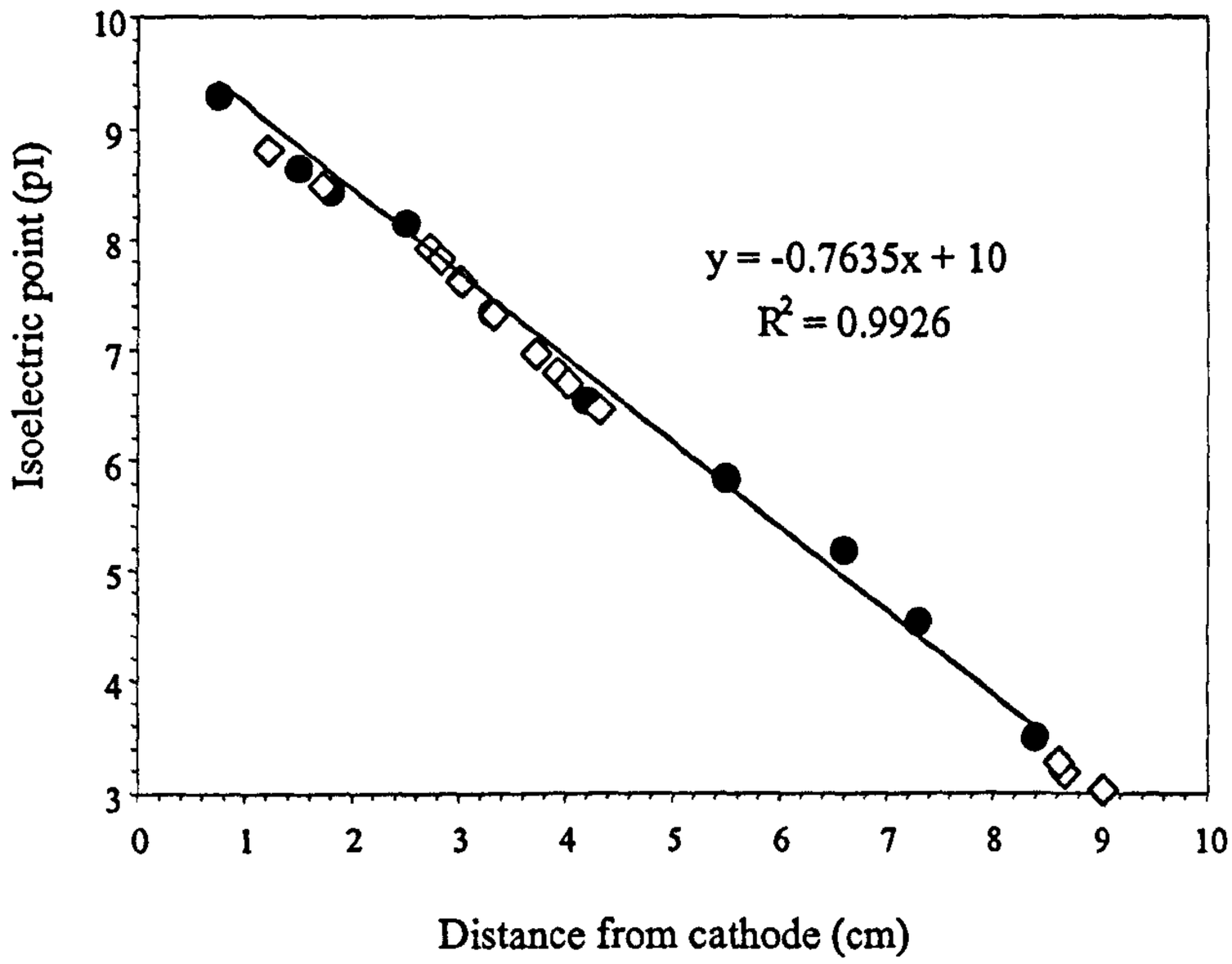


Figure 5.2. Calibration of isoelectric points by linear regression of pI values of marker proteins against distance travelled from the cathode. Dark points are the marker proteins, and open diamonds, protein bands from *C. viridis* venom.

5.2.1 Pattern of venom variation

The pattern of venom variation in *Crotalus viridis* was determined using principal coordinate analysis (PCO), specifying the Gower General Similarity Coefficient. When applied to two-state characters, Gower's coefficient effectively becomes a

Jaccard Coefficient, which omits negative matches, and weights matches more heavily than mismatches (Sneath and Sokal, 1973).

5.2.2 Hypothesis testing

Three alternative hypotheses (independent variables) are considered for geographical variation in *Crotalus viridis* venom (dependent variable), an historical cause (phylogeny), and two current factors, geographic proximity and biotope.

1) The pattern of geographic variation in venom may reflect phylogeny.

Intraspecific variation in venom may be a function of phylogeny, hence similarities in venom properties among populations of a species suggest recent common ancestry (e.g., Githens and George, 1931; Jiménez-Porras, 1964; Tu and Adams, 1968; Foote and MacMahon, 1977; Mandelbaum, 1989; Daltry *et al.*, 1996a; Daltry *et al.*, 1997). In this scenario, phylogenetically closer populations are predicted to secrete more similar venom than phylogenetically distant populations. This was found to be the case among island populations of tiger snakes (*Notechis ater* and *N. scutatus*), in which the degree of variation in venom composition was related to the length of time of isolation on different islands (Williams *et al.*, 1988). As described in Chapter 4, matrices were constructed using the same data set and procedure as for morphology. Matrices of dissimilarity coefficients were constructed from patristic distances (representing phylogeny) between OTUs, extracted from the mitochondrial phylogeny in PAUP (e.g., Thorpe, 1996). Sequence information was available for most of the individuals included here, and where lacking, mitochondrial haplotypes were assigned according to locality.

2) Venom variation is a function of geographic distance between localities.

The opportunity for exchange of venom-coding genes is predicted to be higher between spatially close populations, which are consequently predicted to produce more similar venom than remote conspecifers (e.g., Tu and Adams, 1968). Furthermore neighbouring populations are more likely to share a similar biotic and abiotic environment, potentially resulting in similar unspecified selection pressure for a particular venom type (Daltry *et al.*, 1996a).

3) Venom variation reflects biotope (diet)

The pattern of venom variation reflects biotope, since predator and prey species occupy the same vegetation zones, which are subjected to similar climatic conditions.

The most widely held view is that venom variation is associated with diet and feeding mechanisms, and so diet might represent a selective force shaping venom composition (Thomas and Pough, 1979; Russell, 1983; Mackessy, 1988; Daltry *et al.*, 1996a; Kardong, 1996; Daltry *et al.*, 1997). Being able to subdue and kill prey reduces the hazards of holding onto relatively large prey capable of self-defence (Meier and Stocker, 1995). Furthermore, venom enhances digestion, reducing the risk of putrefaction after ingestion, particularly in species living in cooler climes, such as higher altitudes or latitudes (Thomas and Pough, 1979; Kochva *et al.*, 1983). Geographic variation in venom as a function of diet has been noted in *Calloselasma rhodostoma* by Daltry *et al.*, 1996a, and in *Trimeresurus stejnegeri* by Creer, 2000).

Diet is not tested as a causal hypothesis in the present study, however, for several reasons. First, the sample size of individual *C. viridis* containing dietary items is small (n=85), because most of the museum specimens examined had empty stomachs. Also, the majority of samples come from Pacific coast animals (82%). Second, there does not appear to be geographic variation in prey items at higher taxonomic levels. Unlike adult *Calloselasma rhodostoma*, which feeds on mammals, reptiles and amphibians (Daltry *et al.*, 1996a), the diet of adult *C. viridis* is predominantly mammalian. Finally, it is hard to distinguish among mammal dietary items, especially when only hair is

available, and which would demand a more fine-grained analysis, which becomes impractical in the present circumstances. The diet data accrued to date is summarised in Table 5.2. Whole lizards were only found in snakes less than 550 cm SVL. Mammals were extracted from both juveniles and adults. Debris found in the colons of a few larger snakes (over 600 mm) may possibly be lizard scales, but the identity needs to be confirmed. Other colonic debris found included claws, which may be mammal or lizards. All the unidentified mammals appeared to be rodents, but these items could not be identified to genus or family level. One bird was extracted from a large male southern Pacific rattlesnake (SVL 957 mm). In total 91 food items were retrieved, bearing in mind some snakes contained more than one item.

Biotope is presented as an alternative prey-related hypothesis. The predator and prey occupy the same general abiotic and biotic environments (biotopes). Therefore, if a correlation exists between venom variation and prey, in the absence of dietary information, one might also predict correlation between venom variation and biotope. In this respect, The same climatic and vegetation data were used as outlined in Chapter 4. The generalised biotope was constructed from the sum of a generalised temperature and rainfall column, and a generalised vegetation column. Note that the two columns were standardised prior to addition, to ensure that both abiotic and biotic categories were weighted equally.

Table 5.2. Dietary items removed from the stomachs or colons of *Crotalus viridis*. The category "unidentified" includes samples of debris consisting of any combination of fur, scales, and lizard or mammal claws. The regional categories are: SER - south and east of Rocky Mountains (*C. v. viridis*, *C. v. nuntius*); AZ - Central Highlands, Arizona (*C. v. cerberus*); GB - Great Basin (*C. v. lutosus*, *C. v. abyssus*); PC - Pacific Coast (*C. v. caliginis*, *C. v. helleri*, *C. v. oreganus*).

Genus	Region				Total
	SER	AZ	GB	PC	
Mammals (total = 61)					
Dipodomys - kangaroo rat	2			1	3
Microtus - voles	1			5	6
Mus - house mouse				3	3
Neotoma - wood rats				1	1
Perognathus - pocket mice				1	1
Peromyscus - long tailed mice				9	9
Rattus - rat				1	1
Reithrodontomys - harvest mice				3	3
Sylvilagus - cottontail rabbit				1	1
Thomomys - pocket gopher				1	1
Unidentified mammal	3		2	27	32
Lizards (total = 29)					
Phrynosoma - horned lizard				2	2
Sceloporus - fence lizard				8	8
Unidentified lizard	4	3	1	11	19
Birds					
Unidentified				1	1
Total	10	3	3	75	91

5.2.3 Pairwise and partial Mantel (matrix correspondence) tests

For all hypotheses, matrices and columns of dissimilarity coefficients were constructed using the programs DFmat350 and DFjoin350 respectively (by R. S. Thorpe). All Mantel tests were performed using B. F. J. Manly's program (modified by Thorpe) DFMant350, specifying 10,000 randomisations. The null hypothesis of no association was rejected if $P<0.05$. In the simultaneous tests, row-wise sequential Bonferroni corrections (Rice, 1989) were applied to all the probabilities obtained to correct for the number of hypotheses for each venom component. Pairwise associations were first tested (Table 5.2). Partial Mantel tests were then conducted, to regress out the effects of intercorrelation between the independent variables. In each

case geographic proximity was partialed out, thus removing bias in distances between samples, given that individuals rather than populations were being used.

All multiple samples taken from one individual but at different times, were compared to account for any temporal variation in venom composition. No variation was found between duplicate venom profiles, however, which were excluded from the final analyses. To avoid the effects of sexual dimorphism, males and females should be treated separately. The sex was not known for all individuals, however, and preliminary pairwise and partial Mantel tests found no association between venom composition and sex, among those specimens where the sex was known. Consequently both sexes were treated simultaneously in the final analysis, which also served to maintain a suitable sample size. Ontogenetic variation was of no concern, since only adult specimens were included in the analysis.

5.3 Results

Thirty or more protein bands were present in each sample, of which 14 were variable. There were numerous basic bands in tightly compressed blocks, which were not easy to read, although many appeared to be common to all individuals. Basic proteins were generally more frequent than acidic. The basic protein bands also tended to warp, most probably resulting from excess salt in the venom samples causing disturbances in the pH gradient (see troubleshooting guidelines in the calibration kit instruction pamphlet, Pharmacia Biotech).

More than one type of venom profile occurs in all localities except Sweetwater County, Wyoming (*C. v. concolor*). At least three venom groupings are apparent from the plot of the first against second principal coordinate scores in figure 5.3. The first group includes venom types from Sweetwater County, which have lower second principal coordinate scores than the other populations. A second group consists of all

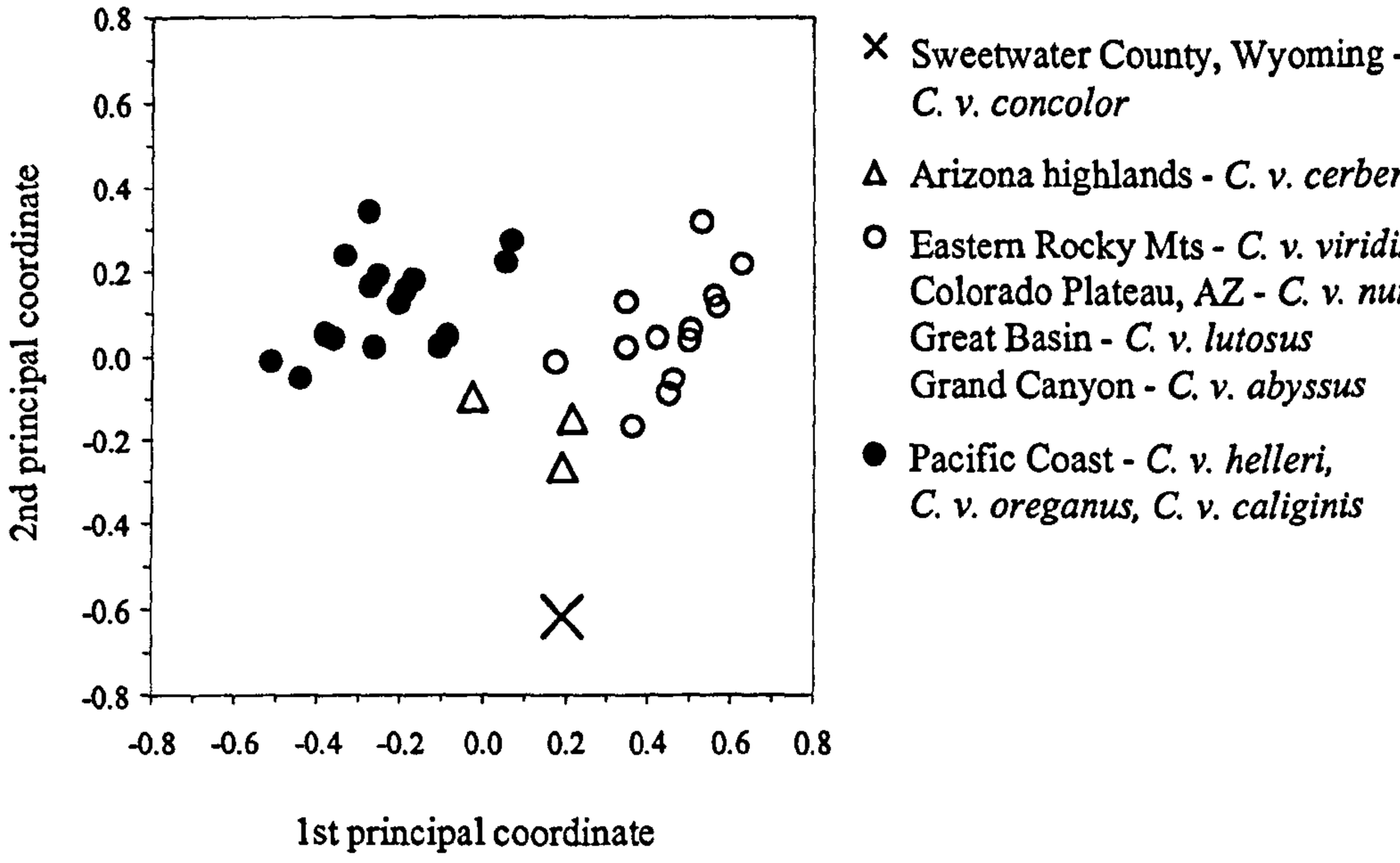


Figure 5.3. Plot of the first and second principal coordinate scores illustrating the pattern of geographic variation in venom composition in *Crotalus viridis*. The first and second axis describe 39.4 % and 17.3 %. of variation respectively.

the Pacific Coast venoms, and there is no distinction between northern or southern forms. The third group includes all other populations, from east of the Rocky Mountains, Arizona, and the Great Basin, although *C. v. cerberus* venoms depart slightly from the main cluster of points. The distribution of principal coordinates groups is shown on the map in figure 5.4. Further PCO of individual clusters did not reveal any further structuring. Results of the pairwise Mantel tests (Table 5.3) revealed significant associations between all three causal hypotheses in the generalised venom type. Individual venom protein bands were predominantly associated with phylogeny and biotope. Only three bands were also related to geographic proximity. When the effects of one or more causal factor are partialed out (Table 5.4), the generalised venom type remains highly significantly associated with all three hypotheses. Individual bands, however, appear to be related to either phylogeny or biotope, but not both (with the exception of one band). None are related to geographic proximity, and three bands showed no significant association with any hypothesis. Note that significant pairwise intercorrelations ($P<0.001$) were also found between all

combinations of causal hypothesis (i.e. phylogeny with geographic proximity, phylogeny with biotope, biotope with geographic proximity).

Figure 5.4. Distribution of *Crotalus viridis* venom-types in North America, categorised according to the results of principal coordinates analysis (see figure 5.3 for scatter plot).

Table 5.3. Mantel coefficients of pairwise association between generalised venom type, and three alternative causal hypotheses. ** indicates significant probabilities at the 1% level, * at the 5% level.

Venom Character		Phylogeny	Geographic proximity	Biotope
Generalised venom		**0.4852****	**0.1779****	**0.6230****
Individual bands pI	3.20	**0.4325****	0.0350	**0.3273****
	3.60	-0.0315	0.0654	**0.3186****
	3.80	**0.1225***	-0.0161	**0.1152****
	6.50	**0.2924****	**0.1720****	**0.4794****
	6.73	**0.1746***	0.1025	**0.3554****
	6.82	**0.2149****	0.0115	**0.1788***
	6.99	**0.1388****	-0.0309	0.0446
	7.35	**0.2717****	0.0831	**0.1087****
	7.55	**0.3148****	**0.1396****	**0.4171****
	7.65	**0.1450****	0.0639	**0.4123****
	7.85	**0.3301****	-0.0307	0.1015
	7.95	**0.1578****	-0.0818	0.0489
	8.52	**0.1366****	**0.1116****	**0.0239****
	8.84	0.1293	-0.0199	**0.1680****

Table 5.4 Absolute standardised partial regression coefficients from Mantel tests of association between generalised venom type and individual venom proteins in *Crotalus viridis* with three alternative causative hypotheses. ** indicates significant probabilities at the 1% level, * at the 5% level. Non significant results are given in reduced font size and shaded grey. Results significant before, but not after sequential Bonferroni correction are shaded grey, not reduced font size, and the significance level for these results indicated by asterisks. - indicates hypotheses that were not significant in the pairwise Mantel tests, and thus excluded from the simultaneous tests.

Venom Character		Phylogeny	Geographic proximity	Biotope
Generalised venom		**0.2001****	**0.0539****	**0.5294****
Individual bands pI	3.20	**0.3633****	-	**0.1230****
	3.60	-	-	**0.3186****
	3.80	0.0844*	-	0.0677
	6.50	0.0332	0.0034	**0.4595****
	6.73	0.0368	-	**0.3761****
	6.82	0.0510	-	0.1393
	6.99	**0.1388****	-	-
	7.35	**0.3080****	-	-0.0645
	7.55	0.1180*	-0.0122	**0.3551****
	7.65	-0.1270*	-	**0.4837****
	7.85	**0.3301****	-	-
	7.95	**0.1578****	-	-
	8.52	-0.0667*	0.0061	**0.3612****
	8.84	-	-	0.1680

5.4 Discussion

The patterns of venom variation in *Crotalus viridis*, as revealed by isoelectric focusing, are similar to those of Young *et al.* (1980) and Foote and MacMahon, (1977), who used similar electrophoretic techniques. Young *et al.* (1980) also noted close similarity of *C. v. abyssus* venom to *C. v. lutosus*. However, Young *et al.* (1980) and Foote and MacMahon (1977) noted only superficial differences between venom profiles of *C. v. concolor* and other populations of *C. viridis*, despite the presence of the high lethality Concolor toxin. In the present study, *C. v. concolor* forms a discrete cluster on its own. However, the venom profiles show that only one band is unique to *C. v. concolor*. When this band is excluded from the principal coordinates analysis, *C. v. concolor* then groups with the largest cluster of points (i.e. including *C. v. lutosus*, *C. v. abyssus*, *C. v. viridis* etc.) suggesting that the difference among these venoms is also superficial. A higher number of different bands was observed in the present study (30+), and by Foote and MacMahon (1977) (33), in contrast to Young *et al.* (1980) who counted 16-21. Band counts are expected to vary between studies due to variations in the electrophoresis conditions, or due to distortions in the gel obscuring the profiles.

Alternative methods such as anion exchange HPLC, and assays testing resulting HPLC fractions for variation in pharmacological activity, are far more sensitive for detecting geographic variation in venom components. These methods have revealed regional variation among venoms of *C. v. lutosus* corresponding to northeast, north-central and southern Utah. In the same study, ELISA tests revealed latitudinal intraspecific variation in haemorrhagic activity (Adame *et al.*, 1990). Interpopulational differences were also found between *C. v. viridis*, *C. v. lutosus* and *C. v. concolor*, in the percentage of myotoxin component, and in the compositional profiles relating to each population (Aird, 1985). SDS-PAGE is another electrophoretic method that can analyse very small quantities of venom and can be carried out in a consistent, repeatable manner. However, this method segregates venom components according to molecular mass, which produces far fewer bands than the separation according to

isoelectric points. Isoelectric focusing, therefore, is considered superior to SDS-PAGE for qualitative comparisons of differences in venom composition, since loss or gain of a given component has a 50% probability in resulting in the clear loss or gain of a pI band, whereas SDS-PAGE bands are liable to merely become more or less dense (Daltry, 1995).

5.4.1 Causes of geographic variation in venom composition

5.4.1.i *Diet and Biotope*

Generally speaking, adult western rattlesnake feed mostly on small mammals with occasional reptiles, birds, and amphibians (Fitch and Twining, 1946; Fitch, 1949; Klauber, 1956; Gannon and Secoy, 1984; Diller and Johnson, 1988; Macartney, 1989). Juveniles rely more on ectothermic prey (lizards and occasionally amphibians), exhibiting a gradual ontogenetic transition to endothermic prey (mammals) (Fitch and Twining, 1946; Fitch, 1949; Klauber, 1956; Mackessy, 1988). Prey-preference is suggested to be constrained by availability of prey in a particular region (Shine, 1987; Wallace and Diller, 1990). For example, *C. viridis* are noted to feed mainly on juvenile ground squirrels (*Spermophilus beecheyi*) in central California (Fitch and Twining, 1946; Fitch, 1949), and southwest Idaho (Diller and Johnson, 1988), while in British Columbia, the main prey items include voles (*Microtus*), deer mice (*Peromyscus*), and northern pocket gophers (*Thomomys talpoides*) (Macartney, 1989). Variation in body size may also influence which size of prey is taken (Wallace and Diller, 1990). Specialisation in prey is reported in areas where a large prey type, for example *Spermophilus*, is abundant, but specialisation decreases in areas (or at ages) where snakes are restricted to eating smaller prey species (Wallace and Diller, 1990). There does not appear, therefore, to be an inherent condition for prey 'choice' in *C. viridis*, suggesting that specialisation is more likely to be a passive response to variation in local availability of prey types.

The question arises to what extent the variation present in the venoms of *C. viridis* (e.g. homologues of myotoxins and PLA_2s) is incidental, or the consequence of adaptation resulting from forced specialisation related to prey availability. Prey immunity to venom provides strong evidence of the potential for an evolutionary arms race to develop between predator venom composition and prey immune system. *Crotalus viridis oreganus* in the western Sierra Nevada foothills specialises on feeding on the California ground squirrel (*Spermophilus beecheyi*), which constitutes about 69% of the rattlesnakes' diet (Fitch, 1948). The squirrels show marked intraspecific variation in resistance to envenomation by *C. v. oreganus* in this region, with squirrels from areas with higher rattlesnake densities being nearly 3 times more resistant than squirrels from areas with low rattlesnake densities. Moreover, blood sera of ground squirrels from habitats with high rattlesnake abundance inhibited *C. v. oreganus* venom more effectively than venoms from both *C. v. viridis* and *C. atrox*. This variation has been explained as an evolutionary response to a long-term association between predator and prey. Squirrels from areas of lower rattlesnake densities do not share the same level of resistance against venom (Poran *et al.*, 1987; Biardi *et al.*, 2000).

Another major consideration is the ontogenetic shift in concentrations of certain components. Ontogenetic variation also has dietary implications. Juvenile venoms of *Crotalus viridis* have far higher toxicity, for example, due to the presence of higher levels of PLA_2s, suggesting an adaptation for killing reptile prey more rapidly (Mackessy, 1988). Given that the adult prey is predominantly mammalian, prey specificity is perhaps less important in terms of venom evolution, than the efficacy of particular venom components to fulfil specific pharmacological functions. Research on the function of specific components supports this idea. Following a bite from *C. viridis*, laboratory mice failed to take a single step, and exhibited hyperextension of the hind limbs characteristic of myotoxic poisoning. It is suggested, therefore, that the biological significance of the small (non-neurotoxic) myotoxins in causing severe muscle necrosis, is to halt the flight of prey almost instantaneously (Ownby *et al.*, 1988).

A recent hypothesis states that venomous snakes use "overkill" to immobilise prey, based on the observation that relatively large amounts of venom are injected into prey, often exceeding 100-1000 times the lethal dose in lab mice. This action supposedly compensates for variation in prey susceptibility. Thus, natural selection for increased potency in toxins is dismissed, on the grounds that there is no apparent need for evolution of more potent venom in response to increasing prey resistance against certain toxins (Mebs, 2001). This statement is highly generalised, given both the broad range in prey resistance, and our lack of knowledge of the exact manner in which snakes use venom for prey immobilisation and digestion.

In a single strike, *Crotalus v. viridis* were found to inject on average 16 mg of venom into deer mice (*Peromyscus*), a natural prey item of wild prairie rattlesnakes. Experiments in which deer mice were artificially dosed with venom, showed that a 5 mg dose of venom would kill a deer mouse in about 268 seconds. Time to death was considerably shorter (120-179 seconds) with higher doses (10-25 mg), although the actual time to death did not show a trend with increasing doses. That less than 16 mg of venom effectively causes death within a short time period might represent a good example of "overkill". However, a more plausible explanation is that of "bet-hedging". The snake can neither gauge the amount of venom required to kill the prey, nor predict the success of a strike. Thus, ejection of excess venom ensures that a lethal dose is administered, which is especially beneficial if the bite itself is ineffective, if only one fang penetrates the prey, or if some venom is lost before the fangs puncture the integument (Hayes *et al.*, 1992). Excess of venom may also provide additive digestive benefits.

Published figures (Lowe *et al.*, 1986) give 44 mg as the mean venom yield in *C. v. viridis*. When this figure is divided by the mean amount ejected in one strike, 16 mg, it is immediately apparent that there is actually only enough venom available to kill 3 *Peromyscus*. Deer mice are quite small (approximately the size of a house mouse), therefore more venom might be required to kill larger prey items, and more venom may be used up if the snake has to make repeated strikes to subdue its prey. Juveniles

and adults of both *C. v. oreganus* and *C. v. viridis* have been shown to be capable of metering venom in relation to size of prey (Hofman *et al.*, 1991; Hayes *et al.*, 1992, 1995; Hayes, 1995). The snakes, therefore, most likely possess just the amount of venom required for subduing just two or three prey items, or less, hence venom is not secreted in great excess, and the use of this venom does not realistically seem like "overkill".

Poran *et al.* (1987) recorded no deaths in ground squirrels (*Spermophilus beecheyi*) from venom resistant areas, administered intramuscular injections of up to 40 mg/kg of *C. v oreganus* venom. Death in squirrels from non-venom-resistant areas occurred around doses of 15 mg/kg and above. These results suggest an approximate LD_{50} of 15 mg/kg in non-resistant squirrels, and over 40 mg/kg in resistant squirrels. The mean venom yield in an adult *C. v. oreganus* is about 90 mg (Lowe *et al.*, 1986), and the weight of ground squirrels in Poran *et al.*'s study ranged between 504-757 g (females) and 630-1140 g (males). Therefore, a northern Pacific rattlesnake, with a mean venom yield of 90 mg, would require at least 20 mg of venom to kill a small to medium (500 g) ground squirrel from the resistant area in several hours, via an intramuscular bite. This suggests the snake has enough venom to kill 4 to 5 squirrels in total. However, in order to kill the prey rapidly (i.e. in a few minutes) as would normally happen in the wild, several LD_{50} doses would be required. This suggests, therefore, that the snake actually has just about the amount of venom it needs for one meal.

The idea of "overkill", therefore, should be treated cautiously, since published LD_{50} values in laboratory animal models may not reflect lethality in natural prey, especially if natural prey exhibits resistance to the venoms. The mean venom yield will also vary according to the size of the snake, and the time to replenish stocks between feeds. Venomous snakes probably inject excessive quantities of venom in one strike to ensure subjugation of prey. Moreover, the physiological location of envenomation is particularly important. Although intravenous envenomation generally leads to death much faster than intraperitoneal injection, the action of venom injected into muscle is far less efficient (as illustrated in the ground squirrel experiments described above),

and most snake bites are subcutaneous or intramuscular. The mass of venom ejected, therefore, may not be a useful correlate of envenomation success, whereas the measurable effects of venom on prey may be more meaningful indicators of envenomation (Hayes, 1992). In conclusion, a hypothesis of overkill should not be formulated without an in-depth study of the role of venom in the foraging ecology an physiology of the snake species concerned.

A second question arises why there is a need for ontogenetic change, if the more potent venoms secreted by juveniles act equally efficiently on reptilian and mammalian prey. One possibility is that the juvenile venom is more specifically adapted to dispatch ectothermic prey, but is less effective on mammals than the adult venom. An alternative explanation is that the juvenile venom works more effectively than adult venom on any prey. This type of venom may be necessary to kill cold-blooded prey more rapidly, but might also be more energetically expensive. Hence, the adult venom may be more specifically adapted for a mammalian diet, while being less expensive to produce than juvenile venom. Dietary evidence (presented here) suggests some adult *C. viridis* also consume lizards. The size difference between an adult rattlesnake of larger bodied *C. viridis* and a *Sceloporus* lizard, however, would not warrant a more potent venom specialised for ectothermic prey. On the other hand, much smaller sized adults such as *C. v. concolor*, may still secrete specialised venom, especially since this population is believed to be more reliant on lizard prey in adulthood. Moreover, the smaller prey being consumed by juveniles or smaller snakes, has a greater surface area to volume ratio, hence internal digestion may be less important.

The most plausible explanation is that in juveniles there is a greater priority for killing prey. Juveniles need to maximise capture of prey, whilst minimising the cost and risk of active foraging, since juveniles possess limited resources to withstand long fasting periods, such as aestivation (Andrade *et al.*, 1996). The capture of a prey once located by juveniles could also be ensured by holding the prey after the strike, a strategy noted in juvenile *C. viridis* (Mackessy, 1988), and captive *B. moojeni* (Andrade *et al.*, 1996).

The priority in adult snakes appears to be more concerned with digestion, due to considerable reduction in surface area to volume ratio associated with larger mammalian prey (Pough and Groves, 1983). The increased proteolytic activity noted in some adult venoms (Mackessey, 1988; Furtado *et al.*, 1991; Andrade *et al.*, 1996) increases breakdown of the visceral tissues, enhancing digestion in the stomach (Thomas and Pough, 1979; Pough and Groves, 1983). In adult snakes, improved digestion is advantageous, because the length of time the snake suffers reduced locomotion and defence capabilities after feeding is lessened, and more time may be spent foraging and in reproduction (Andrade *et al.*, 1996).

The significant association of biotope with 50% of individual venom characters also suggests strong ecological selection on venom composition. In the absence of diet information, biotope provides a useful representation of common ecological conditions between predator and prey. Biotope does not account for microclimate or microhabitat, although these more specific environmental conditions are perhaps of less importance if predator or prey are actively foraging, and moving from one microhabitat to another. In light of the above example concerning dietary specialisation, the emphasis is heightened on the importance of intercorrelation of several causal factors in shaping venom composition.

5.4.1.ii *Phylogeny*

The general characteristics of venoms are seemingly characteristic of the major venomous snake families (Moura-da-Silva *et al.*, 1997), which is also true to some extent at the genus level (Bonilla and Horner, 1969). However, more subtle differences at lower taxonomic levels, particularly intraspecific variation (e.g., Jiménez-Porras, 1964; Táborská, 1971; Glenn and Straight, 1978; Adame *et al.*, 1990; Daltry *et al.*, 1996b; Rodrigues *et al.*, 1998) makes phylogenetic associations more difficult to fathom (Berger and Bhatti, 1989). However, the simultaneous Mantel tests revealed that five protein bands (pIs 3.20, 6.99, 7.35, 7.85, 7.95) were strongly

significantly associated with phylogeny, suggesting that phylogeny is a predictor of a part of venom composition, even within this species complex.

5.4.2 Evolution of PLA_2s in *C. v. concolor*

Gene duplication, followed by hypervariability in regions coding for functional domains, and positive selection have been proposed as evolutionary mechanisms giving rise to the gene families coding for PLA_2s, metalloproteinases, and myotoxins (Moura-da-Silva *et al.*, 1997; Nedelkov *et al.*, 1997). As a result, slightly different molecules with distinct toxic activities are formed.

Crotalus viridis concolor is the only population of *C. viridis* known to secrete Concolor toxin, a high toxicity PLA_2 homologue of Mojave toxin or Crotoxin (Glenn and Straight, 1977) in adulthood. High toxicity has also been reported in juvenile venoms of Pacific Coast rattlesnakes (Mackessy, 1988). A homologous neurotoxic component was reported in four adult individuals of *C. v. viridis* (out of 46 populations tested). Three of these cases, from southwestern New Mexico, have been attributed to hybridisation events with *C. s. scutulatus* (Glenn and Straight, 1990). The fourth, from Page, northern Arizona, is believed to have resulted from intergradation with *C. v. concolor*. Captive breeding has also demonstrated the transfer of genes for lethal Mojave toxins between different species (*Crotalus atrox* and *C. scutulatus*) (Aird *et al.*, 1989).

The evidence outlined above suggests Concolor toxin may have arisen through hybridisation events, where the gene for expression of this toxin in the adult has later become fixed in the population. In contrast, the same gene is being lost in other populations of *C. viridis* by random genetic drift. Alternatively, all populations of *C. viridis* could possess either an inactivated form of the gene encoding neurotoxic PLA_2s (refer to earlier sections, this chapter), or very small amounts of PLA_2 in too low concentrations to initiate any marked toxic effects. Closely related PLA_2s are found in

juvenile venoms of *C. v. helleri* and *C. v. oreganus*, which decrease in concentration with age. These components are also believed to contribute to the increased toxicity in the juvenile venoms (Mackessy, 1988). Differences in PLA_2 content among different populations of *C. viridis*, are thus most likely due to differences in gene expression.

Small amounts of Mojave toxin were reported in *C. atrox* venom samples from two counties in Arizona and one county in Texas (Minton and Weinstein, 1986). However, the type of assay techniques employed by Minton and Weinstein (using polyclonal antibodies raised in rabbits) are known to detect other PLA_2 complexes, that are unrelated to the neurotoxins of interest. This highlights the need for rigorous assaying of venom samples with numerous controls, before accepting the presence of a neurotoxic venom component in a given sample (Aird *et al.*, 1989).

5.4.3 Implications for human health

Experimental studies have demonstrated that not all local tissue damage is prevented by antivenom (Ownby *et al.*, 1997). For the last 50 years, only one commercial antivenom has been available in the United States for the treatment of pitviper envenomation, Wyeth-Ayerst antivenin (Crotalidae) polyvalent (ACP) (Consroe *et al.*, 1995; Dart and McNally, 2001). This antivenom is not particularly effective in combating neurotoxic symptoms, particularly if antivenom administration is delayed (Aird *et al.*, 1989). One reason for this is the regional variation in different venom types, as for example in *C. scutulatus* and *C. durissus*. A common complication also associated with ACP is the high incidence of hypersensitivity reactions, occurring in more than 75% of patients treated (Consroe *et al.*, 1995). Within the last year, a new antivenom (Savage Laboratories, Altana Inc.) has become available. CroFab™ (Crotalidae Polyvalent Immune Fab – Ovine). This antivenom is a preparation of ovine monovalent immunoglobulin (Fab) fragments raised in sheep, using the venoms from *Crotalus atrox* (Western Diamondback rattlesnake), *Crotalus adamanteus* (Eastern Diamondback rattlesnake), *Crotalus scutulatus* (Mojave rattlesnake) and

Agkistrodon piscivorus (Cottonmouth or Water Moccasin). The four monovalent antivenoms are combined in the final product (Bailey *et al.*, 1991). This new antivenom minimises the side effects of hypersensitivity experienced with ACP, and in mouse models FabAV is 3.1-9.6 times more potent than ACP for the prevention of lethality in the United States venoms tested (Table 5.5) (Consroe *et al.*, 1995). To date the efficacy of CroFab™ has only been demonstrated in two sets of clinical trials in a total of 42 patients, presenting minimal to moderate, progressive symptoms of envenomation (Dart and McNally, 2001). The ED_{50} (mg antivenin/mg venom) values (not from clinical trials) for *C. v. helleri* are very high compared to other species (Table 5.6), although this is an improvement upon ACP, which was shown in earlier mouse experiments not at all efficacious in treating *C. v. helleri* envenomation (Consroe *et al.*, 1995). Moderately higher doses of CroFab™ are also required to neutralise *C. m. molossus* and *C. scutulatus* venom.

Table 5.5. Effective dose values for the neutralising effects of CroFAB™ against venoms of clinically important species of *Crotalus*, *Sistrurus* and *Agkistrodon*. Reproduced from the Consroe *et al.*, 1995.

Venom	ED_{50}
C. atrox	5
C. adamanteus	8
C. scutulatus	15
A. piscivorus	3
C. h. atricaudatus	7
C. viridis helleri	122
C. m. molossus	25
A. c. contortrix	4
S. miliarius	7
C. h. horridus	6

Although CroFab™ contains antibodies against *C. scutulatus* venom from the Mojave-toxin positive population (type A) (Clark *et al.*, 1997), it cannot be assumed that antibodies raised against Mojave toxin will effectively neutralise slightly varying homologues in other crotalids, such as Concolor toxin. Given that *Crotalus viridis* is

an important cause of snakebite in the United States (Gomez and Dart, 1995), the low efficacy of CroFab™ against *C. v. helleri* is a source of some concern. Rigorous investigation into the distribution of neurotoxic components remains of the utmost importance. Efforts also need to be focused on maintaining public awareness of the potential lethality of some North American crotalid venoms. Mortality statistics in the U.S. are significantly lower than other countries. However, the numerous examples of intraspecific variation in *Crotalus* venoms, including the broad range of myotoxin isomers and homologues (with or without enzymatic activity), and the extremes produced in pharmacological effects, provide a strong argument for ongoing toxicological research.

Chapter 6. General discussion

6.1 Introduction

To date, this study represents the most comprehensive appraisal, using rigorous hypothesis testing, of geographic variation in *Crotalus viridis*. For many years this wide-ranging, polytypic species, has presented a systematic challenge. Previous research has helped to shed some light on the taxonomic affinities of the group. However, these studies either focused on a limited section of the species distribution (e.g. Schneider, 1986), or used molecular methods that have since been superseded by more advanced markers and technologies (e.g. Quinn, 1987), or used inappropriate analyses of morphological data (e.g. Schneider, 1986; Quinn, 1987), or included only small sample sizes (Quinn, 1987). Taxonomic associations have also been tentatively suggested according to similarities in venoms (e.g. Foote and MacMahon, 1977; Young *et al.*, 1980).

The value of a hypothesis testing approach using contrasting data sets (e.g. Cannatella *et al.*, 1998; Herrmann *et al.*, 1999) has been clearly demonstrated. To reiterate, one of the fundamental issues concerning causal investigation of geographic variation, is that the influence of natural selection on morphology may seriously confound the phylogenetic picture (Hillis, 1987; Thorpe *et al.*, 1991; Daltry *et al.*, 1996a; Thorpe, 1996). The results of the molecular, morphological, and venom analyses here, provide an excellent example of this phenomenon in *C. viridis*.

The most striking first impression of *C. viridis* is the marked contrast in colour pattern and body size, between populations (figures 1.1-1.9). This variation is the root of all misconception surrounding racial affinities within the species complex. Typically, the confusion has arisen from the common acceptance of the subspecies categories (Wilson and Brown, 1953; Cracraft, 1989) based on colour pattern, or body size, as assigned or revised by Klauber. However, Klauber did not actually condone use of

such arbitrary categories, and nominated the subspecies reluctantly, merely to simplify reference to the different populations. In fact, he made a number of highly accurate predictions concerning the racial affinities within *C. viridis*, and never denied the continuity between populations, or the existence of gradual, clinal change between them (for example among the Pacific Coast forms, or between Great Basin and Pacific Coast forms). Neither did he deny that some of these subspecies were, indisputably, one and the same (for example *C. v. viridis* and *C. v. nuntius*, or *C. v. caliginis* and *C. v. helleri*) (Klauber, 1930, 1935, 1949, 1956, 1972).

Although distinct colour patterns may partly characterise different populations of *Crotalus viridis*, colour alone is difficult to quantify for the purposes of morphological study (see Chapter 4). As a whole, colour pattern is clearly an adaptation for crypsis in ambush predators such as *Crotalus* (Klauber, 1972; Kardong, 1980; Sweet, 1985; Greene, 1992; Sazima, 1992; Forsman, 1995; Wüster *et al.*, 1997). Hence it cannot be assumed that similarities among populations for a particular colour pattern reflects phylogeny. A typical example is the past tendency to associate *C. v. cerberus* with *C. v. helleri* due to similarities in juvenile colour pattern and increasing melanism in adults, when in fact these populations are quite distinct when other characters are compared (see Chapter 3) (Klauber, 1949).

New evidence from mitochondrial DNA (Chapter 2) corroborates the idea that the conventional subspecies mask and confound the major patterns of phylogeny. The marked divergence between clades east and west of the Rocky Mountains may indicate two separate species, and *C. v. cerberus* demonstrates a long independent history of evolution and may warrant species status (see below). On the other hand, the species complex includes at least two examples, *C. v. nuntius*, and *C. v. caliginis*, where the conventional subspecies merely represent trivial local varieties, without evidence of a long, independent evolutionary history. Thus, one may advocate synonymising these subspecies. The haplotypes of *C. v. abyssus* are polyphyletic, suggesting that this population may be a hybrid form between populations at either end of the Grand Canyon. Taxonomic revisions are discussed in more detail below.

Additional evidence to refute the subspecies categories stems from geographical patterns of morphological variation (Chapter 3), since the distinction between populations is unclear. Moreover, given that morphological variation in *Crotalus viridis* appears to be strongly associated with phylogeny, this serves as yet further evidence against the subspecies categories, since several clades in the mtDNA phylogeny are not reciprocally monophyletic (Chapter 4).

Phylogeny also explained some geographic variation in a few venom components (Chapter 5). However, the overall pattern of variation in venom (figure 5.3) is not entirely congruent with the mtDNA phylogeny, or the pattern of geographic variation in morphology. The main difference concerns less separation of venom types in the PCO. Despite the lack of separation, the groups formed are not nonsensical. The Pacific Coast forms are clearly similar to each other, and *C. v. concolor* separates out. Even so, these results are of comparatively limited use with respect to resolving the systematic problems of the species.

6.2 Systematics of *Crotalus viridis*

A review of the systematics of *C. viridis* is deemed necessary in light of the lack of congruence between the conventional subspecies categories and the molecular phylogeny, as well as the patterns of variation in morphology and to a lesser extent variation in venom composition. A full taxonomic revision of *C. viridis* is not straightforward, and the ultimate status of the different populations will always be tenuous. The main reason for this is that *C. viridis* is a continuum of a number of morphological types connected by intergrade zones of varying proportions (Klauber, 1949). However, the definition of a species (especially widespread, continental forms) will often be obscure due to "the complex and temporally extended nature of species and the process or processes through which they come into existence" (de Queiroz, 1998). Some populations of *C. viridis* are more distinct than others, and the intergrade zones between these better defined populations are narrow. For instance, the

intergrade zone between *C. v. viridis* and the western forms is not believed to be as broad as that between the Great Basin and Pacific forms (Klauber, 1949). Additional complications arise from sexual dimorphism, in that some characters, such as the number of dorsal body or tail blotches, might be more useful in distinguishing between populations of males than females, because the size differences among the different populations are not proportional in males and females.

The general lineage species concept of de Queiroz (1998), however, provides a useful means of reassessing the taxonomic status of the populations in this tricky species complex. De Queiroz (1998) points out that all modern species definitions (see General Introduction) either explicitly or implicitly equate species with segments of population-level evolutionary lineages, hence all species concepts are special cases of the general lineage concept of species. In this respect, the difficulties of species definitions can be overcome if the many alternative species concepts proposed over the last few decades are viewed not as concepts, but as criteria for defining species, whereby each criterion provides a different kind of information about the separation (or lack thereof) between lineages, or it describes a different stage in the divergence of lineages.

The taxonomic revisions of the *Crotalus viridis* complex are therefore considered according to how well the molecular and morphological (and to a lesser extent venom) evidence conforms to the criteria summarised by de Queiroz (summarised in Table 6.1 and modified from Puorto *et al.*, 2001). Where possible, characters are listed that usefully discriminate between populations in the case of both sexes, and characters influenced by sex are highlighted. Another consideration, given the tenuous nature of taxonomic association in this complex, is that no one character absolutely distinguishes among populations (Klauber, 1956; 1972), therefore the discriminating characters for a given taxon should be considered simultaneously, not independently.

Table 6.1. Criteria for the diagnosis of species, summarised by de Queiroz (1998).

Criterion and references	**Requirements & predictions**
1. The isolation and recognition criteria (Mayr, 1975; Paterson, 1985)	Require a reproductive discontinuity between species, i.e. a lack of intergradation; in terms of phenotype, this leads to the prediction that species should be separated by a clear disjunction, at least in the characters used to diagnose them.
2. The niche criterion (Van Valen, 1976)	Relies on the occupancy of a different adaptive zone as the basis for recognising sets of populations as different species.
3. The phenetic cluster criterion (Sokal and Sneath, 1963)	Species are minimally distinguishable as separate clusters in multivariate analyses.
4. The diagnosability criterion (Cracraft, 1989)	Species are diagnosed on the basis of 'unique combinations of primitive and derived characters' (phylogenetic species concept *sensu* Cracraft, 1989); to qualify as separate species, sets of populations must show fixed differences in some character.
5. The apomorphy criterion (e.g., Baum, 1992)	Operationally, represents a more stringent version of the diagnosability criterion, in which populations constituting a species must share a unique derived character state not possessed by other such groups
6. The concordant coalescence criterion (Baum and Shaw, 1995)	Species are characterised by concordant coalescence of gene genealogies, i.e. all gene trees show the alleles or haplotypes present within species to form clades; a related idea is Moritz, 1994 'Evolutionary Significant Units" (ESUs), based on the presence of monophyletic mtDNA haplotype clades within units. ESUs were not regarded as being synonymous with species.

6.2.1 The basal lineages

It is proposed that the three basal lineages, including the *C. v. viridis-C .v. nuntius* clade, the *C. v. cerberus* lineage, and the lineage consisting of the remaining western haplotypes should be elevated to species level.

6.2.1.i *The east-west split*

The eastern lineage, consisting of *C. v. viridis* and *C. v. nuntius* haplotypes should represent one species, named *Crotalus viridis*. This lineage meets with five out of six general lineage species criteria. Across the entire present species range, there is evidence for only limited gene exchange between the eastern and western clades compared to the level of intergradation among others elsewhere in the distribution (Chapter 3), thus conforming to the isolation and recognition criteria (Mayr, 1963; Paterson, 1985). The present analysis showed no evidence of broad intergradation between the eastern and western clades, with the possible exception of some intergradation with *C. v. concolor* in the southeastern corner of Utah (see below). *Crotalus. v. nuntius* and *C. v. viridis* form a cohesive cluster in the canonical variate analysis (except some individuals from San Juan County, Utah – but see below), which supports the phenetic cluster criterion (Sokal and Sneath, 1963), and these populations are morphologically distinct from other populations in a number of characters, including dorsal pattern, and in the number of intersupraocular scales (see species descriptions below), which conforms to the diagnosability criterion (Cracraft, 1989). The "bean-shaped" blotches of the eastern clade contrast with more rounded or diamond-shaped blotches of the western populations. Since the likely sister species of *C. viridis*, *C. scutulatus*, has diamond-shaped blotches, the pattern of the eastern lineage may be regarded as an apomorphy, thus conforming to the apomorphy criteria of Baum (1992). On the other hand, the higher numbers of intersupraocular scales present in the western clade may be characterised as an apomorphy of that clade, since *C. scutulatus* only has two intersupraoculars. Finally, the eastern and western haplotype lineages are reciprocally monophyletic. In combination with morphological divergence, the two lineages thus meet the requirements of the Evolutionarily Significant Units of Moritz (1994), which has *de facto* been treated as a criterion for determining species boundaries (e.g., Patton et al., 1997). As far as this evidence goes, it is also consistent with the concordant coalescence criterion of Baum & Shaw (1995), although this relies on concordant gene phylogenies across multiple, independent genes, whereas only one single linkage unit was assessed in this study.

6.2.1.ii *Crotalus viridis cerberus*

It is proposed that the subspecies *C. v. cerberus* should be elevated to the status of a full species, *Crotalus cerberus*. In the mitochondrial phylogeny, *Crotalus viridis cerberus* forms a distinct sister group to the remaining western taxa, and appears to have a long, independent evolutionary history. This is reflected in the marked contrast in sequence divergence of 2.99-4.17% between *cerberus* and the remaining western taxa, against 0.1-3.67% within the western clade exclusive of *cerberus* (within-*cerberus* sequence divergence 0.15-0.91%) (Table 2.2). These findings, together with the strong evidence that the eastern lineage constitutes a separate species from all western populations, support the concordant coalescence criterion. As far as is known, *C. v. cerberus* is disjunct from other *C. viridis* (Klauber, 1949; Degenhardt *et al.*, 1996 - also see Chapter 3), and the present data show no evidence of intergradation, even with geographically close populations of the eastern clade, supporting the isolation and recognition criteria. The position of *C. v. cerberus* with respect to other populations has not been clarified from patterns of geographic variation in morphology, and it thus fails to meet the phenetic cluster criterion. The reason for this lack of clarity may be due to the difficulties encountered in recording pattern data for *C. v. cerberus* due to melanism. Several juveniles were also included in the morphological study, which have a markedly different pattern from the adults. As mentioned previously, the position of *C. v. cerberus* in relation to the Pacific forms (figures 3.11 and 3.14) is probably misleading with respect to taxonomic affinities. For many years *C. v. cerberus* was included under *C. v. oreganus,* before being given subspecies status later on (Klauber, 1935, 1949). The earlier taxonomic confusion arose from the morphological similarities in colour pattern between *C. v. cerberus*, and the southern Pacific, *C. v. helleri*. There are in fact distinct differences between *C. v. helleri* and *C. v. cerberus* with respect to dorsal blotch shape, pattern of melanism, and scale counts on the snout, including paired loreals (Klauber, 1949). *Crotalus v. cerberus* differs from the eastern forms markedly in colour pattern, and in some scale counts, including a higher number of intersupraocular scales. It is possible therefore, that the phenetic cluster criterion might

be met if some additional characters were investigated, and if pattern was easier to measure through melanism. With respect to the diagnosability criterion, *C. v. cerberus* can be considered distinct in colour pattern, as described above. Apart from colour pattern, it is difficult to find apomorphic morphological characters in *C. v. cerberus* to support the apomorphy criterion, however the fact that the mtDNA haplotypes give rise to a unique clade in the phylogeny demonstrates the presence of apomorphic characters in the DNA sequence for *C. v. cerberus*.

6.2.1.iii *The western clade (excluding* C. v. cerberus*)*

The remaining western haplotypes form a single clade, which should be elevated to the status of a separate species, *Crotalus oreganus*. The mtDNA haplotypes of the western lineage and *C. v. cerberus* are reciprocally monophyletic, meeting the concordant coalescence criteria. Morphologically, although there is some separation between different groups of western populations in the canonical variate plots, the existence of intergrade forms shows that the morphological groupings are not categorically distinct. However, these morphological types are more separate from the eastern types than they are from each other, thus conforming to the phenetic cluster criterion. The western lineage also fits the diagnosability criterion through differences in colour pattern, higher numbers of intersupraocular scales, and other characters already mentioned above. Difficulty arises with respect to meeting the apomorphy criterion in terms of morphological characters, but certain colour pattern or scale characters may be found to distinguish this lineage more clearly in further studies. As mentioned before, mitochondrial DNA sequence characters unique to the western lineage may be considered apomorphies, thus meeting the apomorphy criterion.

6.2.2 The terminal lineages

6.2.2.i *Status of the subspecies* Crotalus viridis nuntius

There is considerable evidence to doubt the validity of the subspecies *C. v. nuntius*. In the mitochondrial phylogeny (figures 2.6-2.7), *C. v. viridis* as a whole is paraphyletic with respect to *C. v. nuntius*, and *C. v. nuntius* does not show a long history of independent evolution relative to the other *C. v. viridis* populations (figs. 2.6-2.7). Furthermore sequence divergence is the same within as between the respective *C. v. viridis* and *C. v. nuntius* clades (Table 2.2). The similarity is emphasised further in that overall branch (Bremer) support for the arrangement of all the *C. v. viridis* haplotypes is very low, requiring only 1-2 steps to collapse those clades.

The validity of the subspecies *C. v. nuntius* is yet more justifiably questionable on discovering that the state boundary between New Mexico and Arizona was (very subjectively) designated as the border between the two subspecies (Klauber, 1935)! The New Mexico and Arizona populations are, without doubt, continuous with each other. The results of the canonical variate analyses also support the contention that these snakes are not clearly distinguishable morphologically (figures 3.12.a-b). Despite splitting these populations into subspecies, Klauber also noted that body size involved a gradual change moving southwards from Montana. The suggestion of clinal variation is also corroborated by the results of the transect analysis using other morphological characters in Chapter 3 (figures 3.18.a-c).

The results do not conform to any of the general lineage species criteria. *C. v. nuntius* is not reproductively isolated from *C. v. viridis*, there is no separation in the multivariate analysis, there are no clear diagnostic or apomorphic characters to distinguish the two races, and there is no reciprocal monophyly of the mtDNA haplotypes, as *C. v. viridis* is paraphyletic. Thus, the subspecies *C. v. nuntius* does not represent an evolutionary lineage of any description and should thus be synonymised with *C. v. viridis*, leaving *C. viridis* monophyletic.

6.2.2.ii *West of the Rocky Mountains*

6.2.2.ii.a Crotalus viridis abyssus

The relationship of the Grand Canyon population to other *C. viridis* is somewhat complicated. Klauber (1972) suggests an intergrade zone between *C. abyssus* and *C. v. lutosus* at the north rim, and between *C. v. nuntius* and *C. v. abyssus* at the south and east rim areas. Lowe *et al.* (1986) also suggest intergradation between *C. v. abyssus* and *C. v. concolor* more or less throughout the upper stretches of the Colorado River in Arizona, northwards to the Utah border.

The present data provide no molecular or morphological evidence to support the existence of a unique evolutionary lineage, *C. v. abyssus*, in the Grand Canyon. The mtDNA haplotypes of *C. v. abyssus* used in this study are polyphyletic between the Great Basin and *C. v. concolor* clades respectively. As shown in Table 2.2, the levels of sequence divergence for the two individuals included in the analyses are closely concordant with the divergences for the respective clades to which they belong. Sequences for both genes from both individuals have been repeated and checked to ensure that the observed split is not due to error. The canonical variate analyses showed that *C. v. abyssus* consistently clusters with *C. v. lutosus* with respect to morphology (figures 3.11-3.13). Klauber also comments that *C. v. abyssus* and *C. v. lutosus* share morphological similarity in some traits.

As is the case with *C. v. nuntius*, *C. v. abyssus* does not meet with any of the requirements to support designation to species status. The Grand Canyon rattlesnakes appear to be a form of *C. v. lutosus*, with intergrade and hybrid forms occurring near points of contact with other populations of the *C. viridis* complex. Klauber's justification for describing this subspecies was entirely founded on the striking difference in colour from other populations, fading of pattern into maturity, and the restricted distribution of this colour morph in the Grand Canyon (Klauber, 1930).

Since the available evidence does not support *C. v. abyssus* as an evolutionary lineage of any description, recognition of this subspecies would be inappropriate.

6.2.2.ii.b Crotalus viridis concolor

The status of *C. v. concolor* is confusing. The mtDNA data unambiguously place *C. v. concolor* as part of the western clade. The results of the canonical variate analysis are not congruent with the molecular phylogeny, and appear difficult to interpret. A few considerations have come to light, however, whilst writing this thesis. Whilst pooling localities for canonical analyses, individuals from northern parts of Utah (Uintah and Grand Counties) appeared to group with individuals from the southeastern corner of Utah (San Juan County), hence these individuals were allocated to the same pool (no. 9). However, there may be good reason to suspect that the PCA is misleading, probably due to the small sample sizes from northern Utah. Individuals from San Juan County, (although accessioned to the museum collection as *C. v. concolor*) are more characteristic of *C. v. viridis* or *C. v. nuntius* in colour pattern and scale counts. It is more likely that the San Juan individuals are in fact *C. v. viridis*, and the northern individuals *C. v. concolor*. This interpretation explains why in the canonical variate plots, group 9 partially overlaps with *C. v. viridis* and *C. v. nuntius*. The points overlapping with *C. v. viridis* and *C. v. nuntius* are the San Juan individuals, while those that depart from the scatter toward the Great Basin forms are the Uintah and Grand County individuals. The other individuals from Sweetwater County, Wyoming also group with the *C. v. lutosus* individuals.

The apparent morphological heterogeneity of *C. v. concolor* may be due to confusion or intergradation with *C. v. viridis* in south-eastern Utah. Clearly more evidence is required. The existence of a clear "*concolor*" mtDNA clade implies the existence of a lineage of *C. v. concolor* from the Grand Canyon, Arizona, to Sweetwater County, Wyoming. The similarity in morphological pattern of *C. v. concolor* with *C. v. lutosus*

and *C. v. abyssus* may be due to convergent evolution in similar habitat, as supported by the mtDNA phylogeny. The unique venom composition of *C. v. concolor* may also support the status of these individuals as a lineage. However, considerably more sampling is required to establish whether the characteristic venom composition of this taxon is found throughout its range.

Additional morphological, molecular, and venom data are clearly required from a greater part of the range of *C. v. concolor*, and from the Grand Canyon, to explain the various incongruences. It would therefore be premature to rethink the taxonomic status of this population. However, given that *C. v. concolor* belongs to the western lineage, the species name would change to *Crotalus oreganus*. At this point in time, the most conservative option may be to retain *concolor* as a subspecies of *Crotalus oreganus*, until further information becomes available.

6.2.2.ii.c The Pacific Coast: C. v. oreganus, C. v. helleri *and* C. v. caliginis *and Great Basin,* C. v. lutosus.

Having addressed *C. v. abyssus* and *C. v. concolor*, it is now pertinent to examine the remaining western populations together. The haplotypes of the clades corresponding to the Pacific Coast (*C. v oreganus*, *C. v. helleri*, and *C. v. caliginis*) and the Great Basin (*C. v. lutosus*) are very similar. The *C. v. helleri* clade will transpose with the *C. v. lutosus* clade according to whether the analysis is carried out on ND4 or Cyt *b* independently, or using the two genes combined. In the combined sequence tree, bootstrap support for the entire Pacific Coast-Great Basin clade is high (97.4), but the support for *C. v. lutosus* as a sister group to *C. v. oreganus* is low (53%).

Morphologically, and with respect to mtDNA, *C. v. caliginis* is clearly a recently derived island form of *C. v. helleri* (Klauber, 1949), and does not represent an island endemic as suggested by Grismer, 1993. Hence *C. v. caliginis* should be regarded as a synonym of *C. v. helleri*. The fact that *C. v. caliginis* occurs on an island immediately

makes the criteria of isolation or recognition inapplicable. There are no diagnostic differences between *C. v. caliginis* and *C. v. helleri* (even body size differences may have more to do with phenotypic plasticity than genetic difference, as captive *C. v. caliginis* can grow to larger sizes than on the island - pers. obs.).

The strong clinal pattern in morphology between *C. v. oreganus*, *C. v. helleri* (and *C. v. caliginis*) also presents a strong argument to synonymise these forms. *Crotalus v. oreganus* and *C. v. helleri* are not reproductively isolated, they show clinal rather than discrete variation in the multivariate analyses, and they are not immediately diagnosable according to any single or group of characters in the middle part of their distribution. Consequently, no obvious apomorphic characters have been identified. A north-south geographical pattern is apparent in the mtDNA phylogeny, with a north-south separation of haplotypes within the *C. v. oreganus* clade in California. The more northerly clade includes haplotypes from Washington and extreme northern California, and the southern clade includes central and part of southern California. The southern clade is further subdivided into regional central and southern haplotype clades. However, the haplotypes of *C. v. helleri* and *C. v. oreganus* are not reciprocally monophyletic, since the distribution of the two haplotype clades overlaps at least between Monterey and San Luis Obispo Counties. *Crotalus v. oreganus* and *C. v. helleri* thus correspond to none of the criteria for species status, or even that of ESU status (Moritz, 1994). The existence of two major mtDNA haplotype clades in Pacific Coast populations suggests past vicariance. However, geographical overlap between the mtDNA clades and clinal variation across the whole range suggests extensive introgression, so that *C. v. oreganus* and *C. v. helleri* must be regarded as a single organismal lineage. This situation is analogous to that of *Bothrops pradoi* and *B. leucurus* described by Puorto *et al.* (in press). Close similarities in venom profiles, with the result that venoms from both northern and southern Pacific rattlesnakes form a distinct cluster in the PCA, provides additional support for the close association of these populations.

The last piece in the puzzle is *C. v. lutosus*, the Great Basin rattlesnake. In the multivariate analysis, these populations almost formed a separate cluster, but there was

clear intergradation with *C. v. oreganus* in the area in which the contact between the forms was sampled (figures 3.3, 3.9). Irrespective of the apparent differences in colour pattern between the two populations, other characters, particularly scale counts, are very similar. Klauber (1930) describes a very broad contact zone between the two populations, in parts of Washington, Oregon, Idaho, and California along the ridge of the Sierra Nevada Mountains, as far south as Mono Lake, or further. Gene exchange is also thought to occur between the Great Basin and the Pacific Coast, through some of the lower altitude mountain passes. Given the similarities between *C. v. lutosus* and *C. v. oreganus*, it is not unreasonable to suggest that these populations are closely allied.

There is no justification to elevate *C. v. lutosus* to species level, because none of the species criteria can be applied. The isolation criterion is not fulfilled, due to the broad intergradation zone with the Pacific Coast forms; the two populations are not completely separate in a multivariate analyses, thus rejecting the phenetic cluster criterion; it is not straightforward to identify intermediates from the contact zone according to colour pattern, hence both the diagnosability and apomorphy criteria are not fulfilled. Neither does the molecular phylogeny agree with the concordant coalescence criterion: the three clades of *C. v. helleri*, *C. v. oreganus*, and *C. v. lutosus* are not reciprocally monophyletic, because, as mentioned earlier, *C. v. oreganus* and *C. v. helleri* (considered to be synonymous) are paraphyletic with respect to *C. v. lutosus*, although this relationship is weakly supported by the bootstrap.

A clear, widespread *C. v. lutosus* clade is formed in the mtDNA phylogeny, suggesting *C. v. lutosus* is an independent evolutionary lineage. However, intergradation occurs at the contact zone with *C. v. oreganus*, hence *C. v. lutosus* is not reproductively isolated. The intergradation zone in Idaho is broad, suggesting considerable gene flow, hence *C. lutosus* is not considered to be a fully independent lineage. Reciprocal monophyly of the Great Basin-Pacific Coast clades is not supported by mtDNA, although the arrangement of the four main clades (*C. v. helleri*, *C. v. concolor*, *C. v. oreganus*, *C. v. lutosus*) is only weakly supported by the bootstrap. Recognition of *C. v. lutosus* as a subspecies or species depends on the balance between evidence for independent

evolution (general morphology, mtDNA clades), and evidence for cohesion with the Pacific Coast populations through genetic exchange in contact zones. In view of the differences in venom and morphology, but taking into account extensive introgression between *C. v. lutosus* and *C. v. oreganus*, it is suggested that *lutosus* should be retained as a subspecies of *C. oreganus*. However, it should be emphasised that understanding *lutosus* as a differentiated lineage on a partially independent evolutionary trajectory is more important than formal nomenclature.

In this instance, the use of the subspecies category might be usefully applied: *lutosus* is a differentiated form, which has evolved in partial independence from the Pacific Coast populations, but nevertheless retains some cohesion with the latter due to the existence of extensive intergradation. In these circumstances, the rank of subspecies may be usefully employed to denote a population in a state of incipient but incomplete speciation, but not yet consistent with the criteria usually employed to diagnose fully separate species. Thus, it is proposed that *C. v. lutosus* should be renamed *Crotalus oreganus lutosus*, and the Pacific Coast forms (*C. v. caliginis*, *C. v. helleri*, C. *v. oreganus*) become the nominate race, *Crotalus oreganus oreganus*. In due course, these categories may be reviewed once mitochondrial haplotypes and other genetic markers have been examined from a wider range of individuals from the intergrade zones.

6.2.3 Summary of taxonomic revisions

1 The subspecies *Crotalus viridis nuntius* should be synonymised with *Crotalus viridis viridis*, and the latter elevated to the status of a monotypic species, *Crotalus viridis*.

2 The subspecies *Crotalus viridis cerberus* should be elevated to species status, *Crotalus cerberus*.

3 All populations west of the Rocky Mountains forming the western lineage, (excluding *C. cerberus*) should be elevated to the status of a full species, *Crotalus oreganus.*

4 The subspecies *Crotalus viridis abyssus* should not be recognised. These forms are most likely *Crotalus viridis lutosus*, and maybe include intergrade or hybrid forms at points where this form meets other taxa.

5 The subspecies *Crotalus viridis helleri and Crotalus viridis caliginis* should be synonymised with *Crotalus oreganus oreganus*.

6 The subspecies *Crotalus viridis lutosus* should be renamed *Crotalus oreganus lutosus.*

7 As a consequence of elevating the western lineage to *Crotalus oreganus*, the subspecies *C. v. concolor* becomes *Crotalus oreganus concolor* by default, pending further investigation regarding the racial affinities of this population.

6.3 Synonymy

The following synonymy represents a partial summary, including names of *Crotalus viridis* that have had some importance in the literature during the last two centuries, and that have been applied to different populations. Synonyms were assigned to the taxa recognised in this study based on their type locality and identity, as discussed in Klauber (1972) and McDiarmid *et al.* (1999).

6.3.1 *Crotalus viridis* (Rafinesque, 1818)

1818 *Crotalinus viridis* Rafinesque, Amer. Month. Mag & Crit. Rev., 4(1): p.41.

1823 *Crotalus confluentus* Say, in Long's Exped. From Pittsburgh to Rocky Mountains, edited by Edwin James, vol 2, p.48, footnote.

1935 *Crotalus confluentus nuntius* Klauber, Trans. Trans. San. Diego. Soc, Nat. Hist., 8(13): p.78.

1936 *Crotalus viridis nuntius* Klauber, Trans. San. Diego. Soc, Nat. Hist., 8(20): p.191

1936 *Crotalus viridis viridis* Klauber, Trans. San. Diego. Soc, Nat. Hist., 8(20): p.191

6.3.2 *Crotalus cerberus* (Coues, 1875)

1866 *Caudisona lucifer* Cope, Proc. Acad. Nat. Sci. Phila. 18: p307

1875 *Caudisona lucifer* var. *cerberus* Coues, Surv. W. of 100th Merid. (Wheeler) Vol. 5, Chapter 5, p606

1883 *Crotalus oregonus* var. *cerberus* Garman, Mem. Mus. Comp. Zoöl, 8(3): p137

1936 *Crotalus viridis oreganus* (part) Klauber, Trans. San Diego Soc., Nat. Hist., 8(20): p191

1949 *Crotalus viridis cerberus* Klauber, Trans. San Diego Soc., Nat. Hist., 11(6): p83

6.3.3 *Crotalus oreganus oreganus* Holbrook, 1840

1840 *Crotalus oreganus* Holbrook, North Amer. Herp., ed. 1, vol. 4, p.115.

1852 *Crotalus lucifer* Baird and Girard, Proc. Acad. Nat. Sci. Phila. 6: p.177

1859 *Crotalus lucifer* Cope, Proc. Acad. Nat. Sci. Phila. 11: p.337

1883 *Crotalus confluentus* var. *lucifer* (part), Cope, Proc. Acad. Nat. Sci. Phila. 35: p.11

1896 *Crotalus confluentus* (part) Boulenger, Cat. Snakes Brit. Mus., 3: p 576

1905 *Crotalus oregonus* Van Denburgh, Proc. Calif. Acad. Sci., ser 3, 4(1): p.18.

1905 *Crotalus helleri* Meek, Field Col. Mus. Zoöl., Ser., 7(1): pub. 104, p.7

1929 *Crotalus confluentus oreganus* (part) Amaral, Bull. Antivenin Inst. Amer., 2(4): p.92.

1936 *Crotalus viridis oreganus* Klauber, Trans. San Diego Soc., Nat. Hist., 8(20): p.191

1949 *Crotalus viridis caliginis* Klauber, Trans. San Diego Soc., Nat. Hist., 11(6): p.90

1949 *Crotalus viridis helleri* Klauber, Trans. San Diego Soc., Nat. Hist., 11(6): p.77

6.3.4 *Crotalus oreganus lutosus* Klauber, 1930

1859 *Crotalus lucifer* Baird, Pac. R. R. Surv. (Williamson), 10(4): p. 10

1875 *Crotalus confluentus* (part) Yarrow, Surv. W. of 100th Merid. (Wheeler), vol. 5, Chapter 1, p.530

1883 *Crotalus confluentus* lucifer Cope, 1859, Proc. Acad. Nat. Sci. Phila. 35: pp.11

1898 *Crotalus oregonus* (part) Van Denburgh, Proc. Amer. Philos. Soc. Nat. Hist. 37(157): p.141

1930 *Crotalus confluentus abyssus* Klauber, Trans. San Diego Soc., Nat. Hist., 6(3): p.114

1930 *Crotalus confluentus lutosus* Klauber, Trans. San Diego Soc., Nat. Hist., 6(3): p.100

1936 *Crotalus viridis abyssus* Klauber, Trans. San Diego Soc., Nat. Hist., 8(20): p.191

1936 *Crotalus viridis lutosus* Klauber, Trans. San Diego Soc., Nat. Hist., 8(20): p.191

Nomenclatural note: the names *lutosus* and *abyssus* were published simultaneously in the same publication by Klauber (1930). Since the two names refer to the same subspecies, and priority cannot be objectively determined, the Principle of the First Reviser applies in accordance with Article 24.2.2 of the International Code of Zoological Nomenclature, 4th edition. The name *lutosus* has been in widespread use for a widely distributed and fairly well characterised taxon, whereas the name *abyssus* has been restricted to a localised population of the same taxon, which, furthermore, may constitute an intergrade population. Consequently, I give priority to the name *lutosus* over the name *abyssus*.

6.3.5 *Crotalus oreganus concolor* Woodbury, 1929

1915 *Crotalus oregonus* (part) Van Denburgh and Slevin, Proc. Calif. Acad. Sci., ser. 4, 5(4): p.109

1929 *Crotalus concolor* Woodbury, Bull. Univ. of Utah, 20(6): p.2

1930 *Crotalus confluentus* decolor Klauber, Trans. San Diego Soc., Nat. Hist., 6(3): p.111

1936 *Crotalus viridis concolor* Klauber, Trans. San Diego Soc., Nat. Hist., 8(20): p.191

1940 *Crotalus viridis decolor* Gloyd, Chi. Acad. Sci. spec. pub 4, p.216.

6.4 Species descriptions

Discriminant function analysis (using BMDP program 7M) was used to determined which characters discriminated between the different populations. No single character exists to reliably distinguish among all populations, but characters used in combination are important.

6.4.1 *Crotalus viridis*

Animals fitting the descriptions of the conventional subspecies, *Crotalus viridis viridis* and *C. v. nuntius* as described in detail by Klauber (1930; 1936; 1949; 1972). Dorsal blotch markings are eliptical in shape, with indentations at the centre of the long-edge of the ellipse, in an anterior-posterior direction. Postocular stripe usually 1 scale wide (Klauber, 1930). Usually 3-4 intersupraocular scales. Occasional individuals have up to 6, whereupon colour pattern is more useful in distinguishing this species from Crotalus oreganus. Generally a greater number of dorsal blotches than the Pacific Coast forms. Great Basin individuals have similar numbers of blotches to *C. viridis*, but distinctly different blotch shape and borders.

6.4.2 *Crotalus cerberus*

A distinct colour pattern as described by Klauber (1949; 1972). Distinguished from *C. oreganus* by two loreal scales (as oppose to one in *C. oreganus*), and a greater number of scales between the prenasal and the first supralabial at the rostral scale (Klauber, 1949). Additional colour pattern characters, include a lower number of dorsal blotches, and fewer blotches becoming bands in the posterior third of the body, than *C. oreganus*. Apart from obvious differences in dorsal colour pattern, this species is also distinguished from *C. viridis* by a higher number of intersupraocular scales, and high melanism of the ventral surface (the venter of *C. viridis* is rarely heavily pigmented).

6.4.3 *Crotalus oreganus oreganus*

Dorsal blotch markings are circular, hexagonal, or diamond-shaped. Dark ground colour and blotches (dark brown, dark grey, or black), some individuals being almost melanistic. Last dark tail ring in snakes from the southern parts of California, and northern parts of Baja California, Mexico, is about twice as wide as the others and ill defined. This band is yellow in juveniles. Northern populations have clearly defined dark tails rings of uniform width. Fewer dorsal blotches than *C. viridis*. Usually between 6 and 8 intersupraocular scales, although some individuals have 3 or 4 (as in *C. viridis*), whereupon colour pattern will be useful in distinguishing the two species. Postocular stripe usually 2-3 scales wide.

6.4.4 *Crotalus oreganus lutosus*

Morphologically distinguished from *C. o. oreganus* by colour pattern. Ground colour light, beige or pale brown. Dorsal blotches dark brown to black (often with light centres). Usually 6 to 8 intersupraocular scales. More dorsal blotches than *C. o.*

oreganus. Usually 3 scale rows between the upper labials and subocular scales. Postocular stripe usually 2-3 scales wide.

6.4.5 *Crotalus oreganus concolor*

The subspecies *Crotalus oreganus concolor* is currently insufficiently defined to allow a description. More data is required to allow a review of this taxon.

6.5 Future research.

The combined evidence from the phylogeny and morphology, and to a lesser extent venom, has been very useful in elucidating, and revising the taxonomic associations of *Crotalus viridis*. The recommended changes, however, should not be considered definitive without further evidence from alternative data sets (Thorpe, 1996; Cannatella *et al.*, 1998). The mode of evolution of mitochondrial DNA, for example, does not reflect that of nuclear DNA. Nuclear gene phylogenies more appropriately address systematic questions at higher taxonomic levels (e.g. Gianassi *et al.*, 2001), due to the slow rates of base substitution in nuclear genes compared to mitochondrial genes. However, amplified fragment length polymorphism (AFLP, Vos *et al.*, 1995) provides an alternative approach to nuclear phylogeny at the intraspecific level (e.g. Mueller and Wolfenbarger, 1999). Genomic nuclear DNA is cut into fragments using site specific endonucleases, and selected three base (or two base) primers ligated to the ends of the fragments. The fragments are then amplified using PCR, and separated by electrophoresis, producing genotype banding patterns of individual organisms. This genotyping method provides evidence of gene flow within or between populations, but cannot be used for phylogenetic reconstruction, since two character state (presence or absence of bands) similarity coefficients are used. An advantage of AFLP is that the potential effects of lineage sorting are avoided (Moore, 1995; Maddison, 1997; Slowinski and Page, 1999).

Electrophoretic approaches to investigating venom variation have been criticised, since individual venom bands cannot be identified, and may have no adaptive value (see Sasa, 1999a; Sasa, 1999b; Wüster *et al.*, 1999b; Mebs, 2001). There is evidence to refute these criticisms, since studies of venom variation by isoelectric focusing have revealed significant associations with diet (Creer, 2000; Daltry *et al.*, 1996a), and in the present study, phylogeny and biotope (see Chapter 5). Additional information regarding specific venom proteins may be gained from mass spectrometry studies. Matrix Assisted Laser Desorption Ionisation - Time of Flight Mass spectrometry (MALDI-TOF-MS) has been used to analyse arachnid venoms (Escoubas *et al.*, 1999) and venom from the Bamboo pitviper, *Trimeresurus stejnegeri* (see Creer, 2000 for details and descriptions of the method). MALDI-TOF a sensitive approach for direct, mass identification of components from complex mixtures (Mirgorodskaya *et al.*, 2000). The distinct lack of fragmentation coupled with the relatively high sensitivity of the technique, means that all peaks (except those relating to the matrix) can be correlated to single components within a given mixture. Therefore, results may be generated rapidly, without any prior chromatographic separation of components (Creer, 2000). MALDI-TOF information has just recently been obtained for *Crotalus viridis*. A further understanding of evolution of venom composition may also be gained from studies of venom coding genes (Moura-da-Silva *et al.*, 1997).

This study has also demonstrated the value of morphology towards a better understanding geographic variation in *C. viridis*. This highlights the fact that traditional morphological data still form an integral part of the field of systematics, despite the surge in popularity for modern molecular approaches. In this respect, this study should not represent an end point in amassing morphological data for *C. viridis*, since other characters not considered here may yet be found to be important, both diagnostically, and in testing causal hypotheses of geographic variation.

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Appendix I. Details of samples used to reconstruct phylogeny.

The field references are used by C. E. Pook and numbers in square brackets are the localities codes used in Figure 2.2. The second reference includes personal credits, institution voucher/accession numbers, or captivity reference codes: **UTEP**, University of Texas El Paso; **ASDM**, Arizona-Sonora Desert Museum; **KA**, Kyle Ashton; **MC**, Mike Cardwell; **WH**, Bill Hayes; **IB**, Instituto Butantan, São Paulo; **RM**, Ross MacCulloch; **TM**, Tom Moisi; **MVZ**, Museum of Vertebrate Zoology, Berkeley; **ROM**, Royal Ontario Museum, Toronto; **WCS**, Wade Sherbrooke; **LS**, Lee Simons; **SW**, Steve Werman; **UCWB**, University of Wales, Bangor; **CEP**, personal collection C. E. Pook; **WW**, W. Wüster. For GenBank accession numbers see Pook *et al.* 2000. The outgroups used were *Crotalus durissus terrificus*: Brazil: São Paulo: Pindamonhangaba, WW 136 IB 55600-1 and *Crotalus scutulatus scutulatus*: USA: New Mexico: Doña Ana Co. C. E. Pook field ref. 050 UTEP: CRH 153.

Ref	Taxon	Locality / [Locality code]		Grid Reference	Ref 2
001	*oreganus*	Snively Jctn, Tehama Co., CA	[3]	40.317 122.234	LS
002	*oreganus*	Snively Jctn, Tehama Co., CA	[3]	40.317 122.234	LS
003	*oreganus*	Snively Jctn, Tehama Co., CA	[3]	40.317 122.234	LS
007	*helleri*	San Jacinto Mt., Riverside Co., CA	[11]	34.000 117.000	WH CVH65
008	*helleri*	San Jacinto Mt., Riverside Co., CA	[11]	34.000 117.000	WH CVH63
009	*helleri*	San Jacinto Mt., Riverside Co., CA	[11]	34.000 117.000	WH CVH91
010	*abyssus*	Coconino Co., AZ	[16]	36.000 113.500	TM
011	*lutosus*	Las Vegas, Clark Co., NV	[15]	36.120 115.700	TM
012	*nuntius*	Coconino Co., AZ	[16]	35.184 111.367	TM
014	*caliginis*	South Coronado Island, Mexico	[12]	32.417 117.250	TM
015	*viridis*	Animas, Hidalgo Co., NM	[19]	31.900 108.750	TM
016	*cerberus*	Graham Mts., Graham Co., AZ	[17]	32.584 109.834	TM
017	*oreganus*	San Luis Obispo Co., CA	[7]	35.250 120.500	TM
020	*lutosus*	Lava Beds Natl. Monument, Modoc Co., CA	[1]	41.750 121.567	MC 87083001
021	*lutosus*	Washington Co., UT	[28]	37.250 113.500	MC
023	*he'or*	Paso Robles, San Luis Obispo Co., CA	[7]	35.634 120.717	MC 97052501
025	*oreganus*	Pullman, Whitman Co., WA	[13]	46.700 177.000	WH CVO13
026	*oreganus*	Summit Rd, Santa Cruz Co., CA	[6]	37.000 122.000	WH CVO03
039	*helleri*	San Bernardino Co., CA	[9]	34.100 117.150	WH CVH18
044	*viridis*	Eddy Co., NM	[22]	31.900 106.250	ASDM 92290
045	*abyssus*	Grand Canyon, Coconino Co., AZ	[16]	36.000 113.500	ASDM95069
046	*viridis*	Fort Bliss, El Paso, TX	[24]	31.900 106.250	ASDM92302
047	*cerberus*	Mt. Lemmon, Pima Co., AZ	[18]	32.450 111.050	ASDM91076

...continued

Ref	Taxon	Locality / [Locality code]		Grid Reference	Ref 2
048	*viridis*	State Hwy 9, Dona Ana Co., NM	[20]	31.817 106.734	UTEP17625
055	*viridis*	24 mi N of Craig, Moffat Co., CO	[32]	40.750 107.500	KA
056	*viridis*	24 mi N of Craig, Moffat Co., CO	[32]	40.750 107.500	KA
060	*viridis*	Wycross Ranch, Laramie, Laramie Co., WY	[31]	41.400 105.200	KA
068	*concolor*	Flaming Gorge Res., Sweetwater Co., WY	[30]	41.134 109.528	KA
069	*concolor*	Flaming Gorge Res., Sweetwater Co., WY	[30]	41.134 109.528	KA
070	*concolor*	Flaming Gorge Res., Sweetwater Co., WY	[30]	41.134 109.528	KA
071	*concolor*	Flaming Gorge Res., Sweetwater Co., WY	[30]	41.134 109.528	KA
073	*viridis*	Fort Bliss Military Res.,Otero Co., NM	[21]	32.250 106.000	UTEP15871
074	*viridis*	Corralitos Ranch Rd, Dona Ana Co., NM	[20]	31.817 106.745	UTEP17518
076	*viridis*	State hwy 21, Colfax Co., NM	[23]	36.340 104.834	UTEP14754
079	*helleri*	Ko Pah Mts., San Diego Co., CA	[10]	32.573 116.944	UTEP17519
081	*viridis*	Farm Rd 119 Sherman Co., TX	[27]	36.250 101.800	UTEP15872
088	*oreganus*	border with Oregon, Siskiyou Co., CA	[2]	41.750 122.617	LS
093	*viridis*	Geraldine, Chouteau Co., MT	[33]	47.500 110.000	ROM 16109
094	*viridis*	Geraldine, Chouteau Co., MT	[33]	47.500 110.000	RM 2053
096	*helleri*	Pearblossom, Los Angeles Co., CA	[8]	34.634 118.000	ROM 19757
097	*helleri*	Saugus, Los Angeles Co., CA	[8]	34.634 118.000	ROM 19656
098	*helleri*	Pyru Creek, Los Angeles Co., CA	[8]	34.634 118.000	RM 1967
101	*lutosus*	Ichthyosaur Monument, Nye Co., NV	[14]	38.884 117.580	ROM 1
102	*oreganus*	Berkeley, Alameda Co., CA	[4]	37.917 122.250	ROM 19764
105	*oreganus*	Corral Hollow, San Joaquin Co., CA	[4]	37.634 121.550	MVZ 150246
106	*oreganus*	Dwight Way, Alameda Co., CA	[4]	37.634 121.550	MVZ 150248
114	*oreganus*	Stanislaus Co., CA	[5]	37.417 121.384	MVZ 150250
116	*viridis*	Animas, Hidalgo Co., NM	[19]	31.900 108.750	HWG 2084
117	*cerberus*	Pinaleno Mts., Graham Co., AZ	[17]	32.592 109.850	WCS
119	*helleri*	Otay Mesa, San Diego Co., CA	[10]	32.573 116.944	CEP
120	*helleri*	Otay Mesa, San Diego Co., CA	[10]	32.573 116.944	CEP
121	*helleri*	Otay Mesa, San Diego Co., CA	[10]	32.573 116.944	CEP
125	*lutosus*	Red Butte, Salt Lake Co., UT	[29]	40.500 112.000	CEP
126	*lutosus*	Red Butte, Salt Lake Co., UT	[29]	40.500 112.000	CEP
127	*cerberus*	Stockton Pass Wash, Graham Co., AZ	[17]	32.592 109.850	UCWB
129	*viridis*	Old highway 666, Apache Co., AZ	[42]	34.191 109.343	UCWB
130	*viridis*	Old highway 666, Apache Co., AZ	[42]	34.191 109.343	UCWB
131	*nuntius*	Coconino Co., AZ	[16]	35.192 111.374	UCWB
132	*nuntius*	Coconino Co., AZ	[16]	35.118 111.031	UCWB
133	*cerberus*	Hwy 89, nr. Payson, Gila Co., AZ	[41]	34.284 111.334	UCWB
138	*oreganus*	Sequoia Natl. Pk,Tulare Co.,CA	[35]	36.667 118.500	CAS 206480
139	*oreganus*	Ford Ord, Monterey Co., CA	[39]	36.667 121.767	CAS 202983
140	*oreganus*	Tahoe Natl. For., Sierra Co., CA	[36]	39.500 120.950	CAS 202876
141	*oreganus*	Tahoe Natl. For., Sierra Co., CA	[36]	39.534 120.900	CAS 205951
143	*oreganus*	Sweales Flat Rd., Butte Co., CA	[37]	39.434 121.400	CAS 205205
145	*oreganus*	Near Beckworth, Plumas Co., CA	[34]	50.255 120.367	CAS 206022
150	*lutosus*	Hanging Rock Canyon, Washoe Co., NV	[40]	41.517 119.450	CAS 202955
151	*concolor*	Hwy 139, Rio Blanco Co., CO	[30]	40.000 108.750	SW

Appendix II. Laboratory protocols for DNA extraction, PCR and sequencing

DNA PROTOCOLS

DNA extraction from animal tissue

Tissue storage

Blood stored in cell lysis buffer - 2.0% SDS; 100 mM Tris pH 8.0; 0.1 M EDTA pH 8.0

Muscle liver heart - 80% ethanol

DNA template preparation

1 **Cell lysis:** Incubate 10-20 mg of chopped tissue at 65 °C for 15 minutes in 600 µl of cell lysis buffer.

2 **Protein digestion:** Incubate homogenised tissue (from stage 1) *or* 150 µl of blood sample in 400 µl of ultrapure water, overnight at 55 °C with Proteinase K solution (3 µl of 20 mg ml^{-1}).

3 **RNA digestion:** Add 3 µl Rnase (10 mg ml^{-1}) to cell lysates, and incubate at 37 °C for 1 hour.

4 **Protein precipitation:** Cool samples on ice, add 200 µl ice-cold 5 M ammonium acetate solution. Mix thoroughly by inversion and keep at –80 °C for 1 hour.

5 **Pellet proteins by centrifugation** for 20 minutes at 13 000 rpm.

6 **DNA precipitation:** Transfer supernatants to clean 1.5 ml Eppendorfs containing 600µl of ice-cold isopropanol (take care not to dislodge the protein pellets). Mix thoroughly by inversion. Pellet DNA by centrifugation at 13 000 rpm for 20 minutes. Wash DNA pellet in 70% ethanol, and discard ethanol after 15 minutes centrifugation at 13 000 rpm. Invert Eppendorf tubes on absorbent tissue and allow DNA to air dry for 2 hours, or until no trace of ethanol can be seen.

7 **DNA elution:** Elute DNA in 25 µl of TE pH8.0 buffer.

8 **DNA quantification:** Electrophorese 5µl of template DNA through a 1% agarose minigel (1 g agarose per 100 ml TBE buffer + 1 µl per 20 ml ethidium bromide10 mg ml^{-1}) alongside lambda quantification standards of known concentration (ranging from 15 ng to 500 ng). Adjust dilutions to standardise concentration of all samples.

Double-stranded amplification of mitochondrial cytochrome b and ND4 genes using polymerase chain reaction (PCR) (Saiki, 1988).

Cytochrome b primers:

703Bot L-strand (modified from Kocher *et al.*, 1989)

5'-TCA AAC ATC TCA ACC TGA TGA AA-3'

H-strand modified version of primer MVZ 16 of Moritz *et al.*, 1992)

5'-GGC AAA TAG GAA GTA TCA TTC TG-3'.

The 5' ends of these primers correspond to positions 14977 and 15735 of the total mtDNA sequence of *Dinodon semicarinatus* (Kumazawa *et al.*, 1998).

ND4 primers:

ND4 primers were primers ND4 and Leu of Arévalo *et al.*, 1994.

PCR amplification mixture

20 mM Tris-HCl

50 mM KCl

0.52 µM primer 1

0.52 µM primer 2

2.5 mM $MgCl_2$

0.25 mM dNTP

2 units Taq DNA polymerase

0.5% DMSO

DNA template at 1 ng per µl

Make up to 50 µl with ultrapure water

Amplification conditions:

Step 1: Denature for 4 min. at 94 °C; Step 2: Denature for 1 min. at 94 °C; Step 3: primer annealing 1 min. at 50 °C; Step 4: primer extension 2 min. at 72 °C; Repeat steps 2-4 for 35 cycles; Step 5: final extension for 3 min. at 72 °C. Cool to 4 °C.

Purification of PCR products

Purification was carried out using the GenElute™ (Supelco) nucleic acid purification kit. If necessary PCR products were concentrated by pooling up to three products per sample.

1 In preparation place GenElute™ spin columns in 1.5ml Eppendorf tubes
Pass 100 µl of TE (pH 8.0) through the column by centrifugation
Discard and the collected TE

2 Electrophorese the whole PCR product into a large 1% agarose gel (2 hours at 120 volts) alongside a 100 base pair ladder marker.

3 Visualise the products on an ultraviolet light box.
Precisely excise bands from the gel, and place labelled GenElute™ columns
Remove the DNA from the agarose by centrifugation at 13 000 rpm (DNA passes through the filter of the spin column and is collected in the Eppendorf below while the agarose is retained on top of the filter in the columns which can then be discarded).

4 **DNA precipitation:** add 10 µl of 3 M NaAc (pH5.2) and 250 µl of 95% ethanol.
Mix thoroughly, and pellet DNA by centrifuging for 30 minutes at 13 000 rpm. Discard supernatant, and wash DNA pellet with 500 µl of 95% ethanol. Centrifuge again for 15 minutes at 13 000 rpm. Discard the ethanol. Invert

Eppendorf tubes on absorbant tissue and allow DNA to air dry for 2 hours, or until no trace of ethanol can be seen. Air dry DNA pellet by leaving the open, inverted Eppendorfs on absorbent tissue for 2 hours, or until all ethanol has evaporated.

For automated sequencing, quantify the amplified DNA and elute in the appropriate volume of ultrapure water.

Single stranded automated sequencing

1 Prepare 10 µl reaction mixtures containing approximately 50 ng PCR template, 0.16 pmol 5' primer (703Bot primer for Cytochrome *b* reactions; ND4 primer for ND4 reactions), and BigDye Terminator Ready Reaction Mix (ABI).

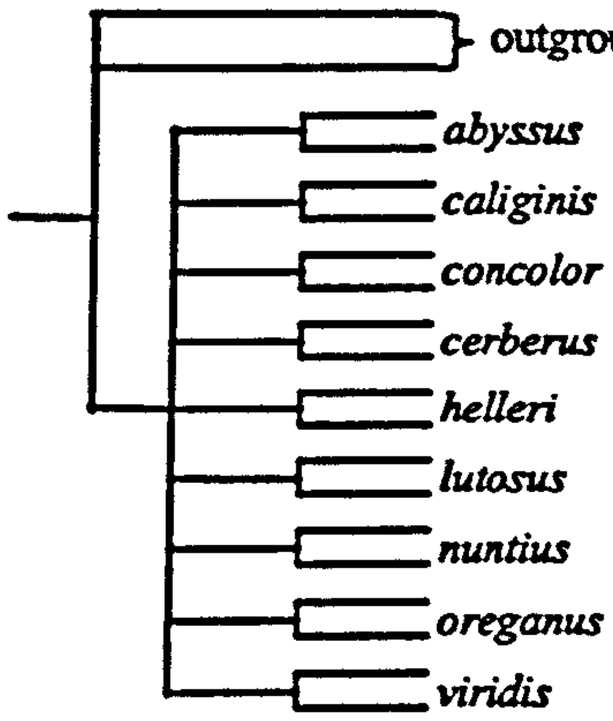
2 Cycle the reaction mixtures in a Thermal Cycler for 30 sec. at 94°C; 50 cycles of 10 sec. at 96°C; 5 sec. at 50°C; 4 min. at 60 °C; cool 4°C.

3 Precipitate the DNA samples using 2M sodium acetate and 100 % ethanol, pellet the DNA, wash in 70 % ethanol, and air dry (as described above).

4 Resuspend the samples in 4 µl of a dextran blue EDTA/formamide loading buffer, and load 1µl of the mixture onto a 5 % Longranger gel (Flowgen) in 1x TBE running buffer.

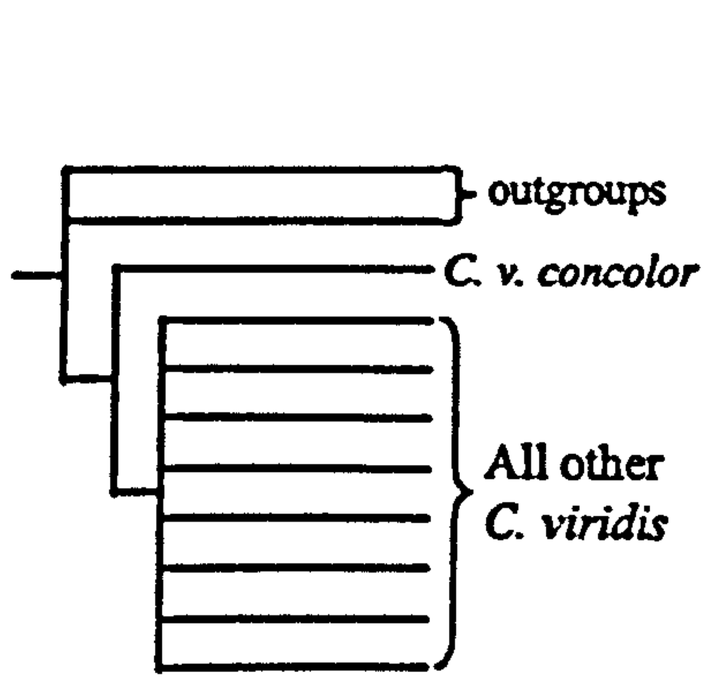
5 Analyse on an ABI 377 DNA Sequencer, generating a chromatogram, and a text sequence.

Appendix III. Phylogenetic hypotheses (Chapter 2, section 2.3.4)



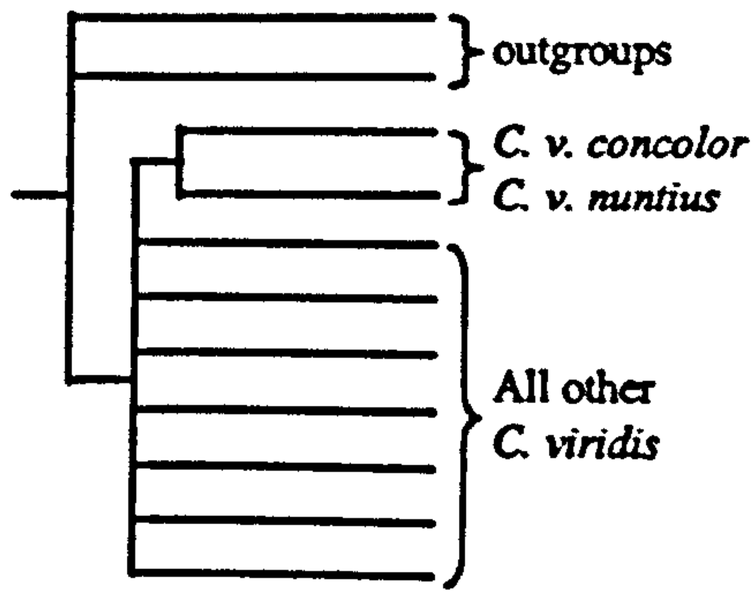
Hypothesis i. Subspecies monophyly

(((*ab*),(*ca*),(*co*),(*ce*),(*he*),(*lu*),(*or*),(*nu*),(*vi*)),*C. durissus*,*C. scutulatus*)



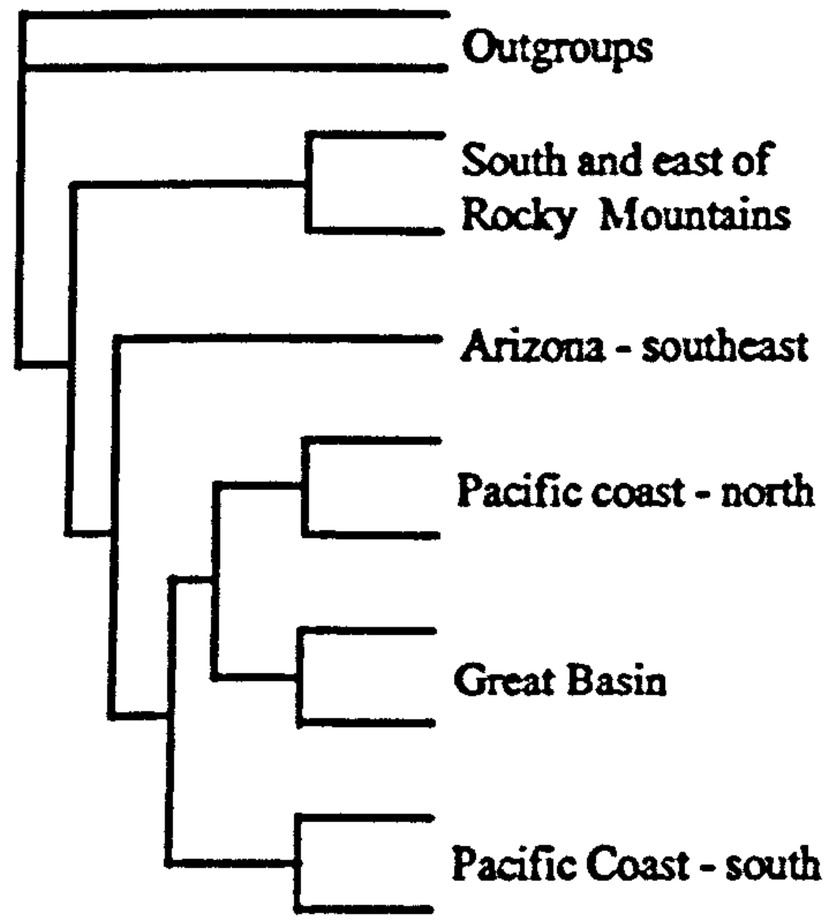
Hypothesis ii. Evolution of Concolor toxin

(((*ab*,*ca*,*ce*,*he*,*lu*,*or*,*nu*,*vi*,)*co*),*C. durissus*,*C. scutulatus*)

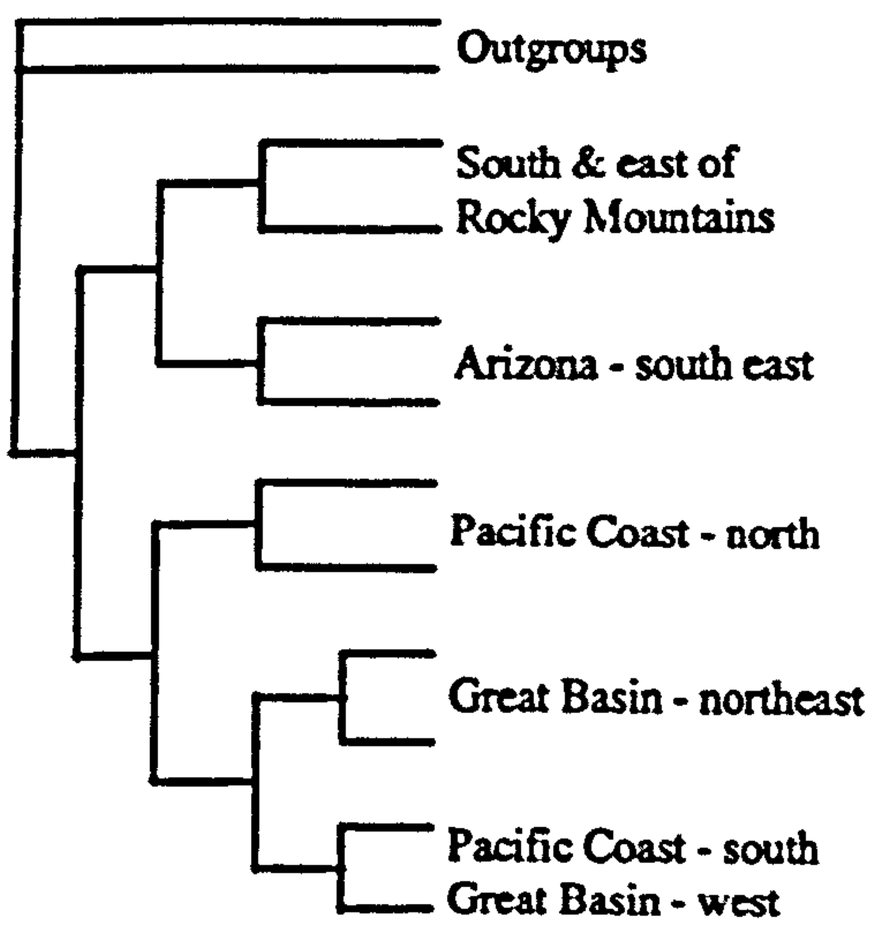


Hypothesis iii. Evolution of small body size

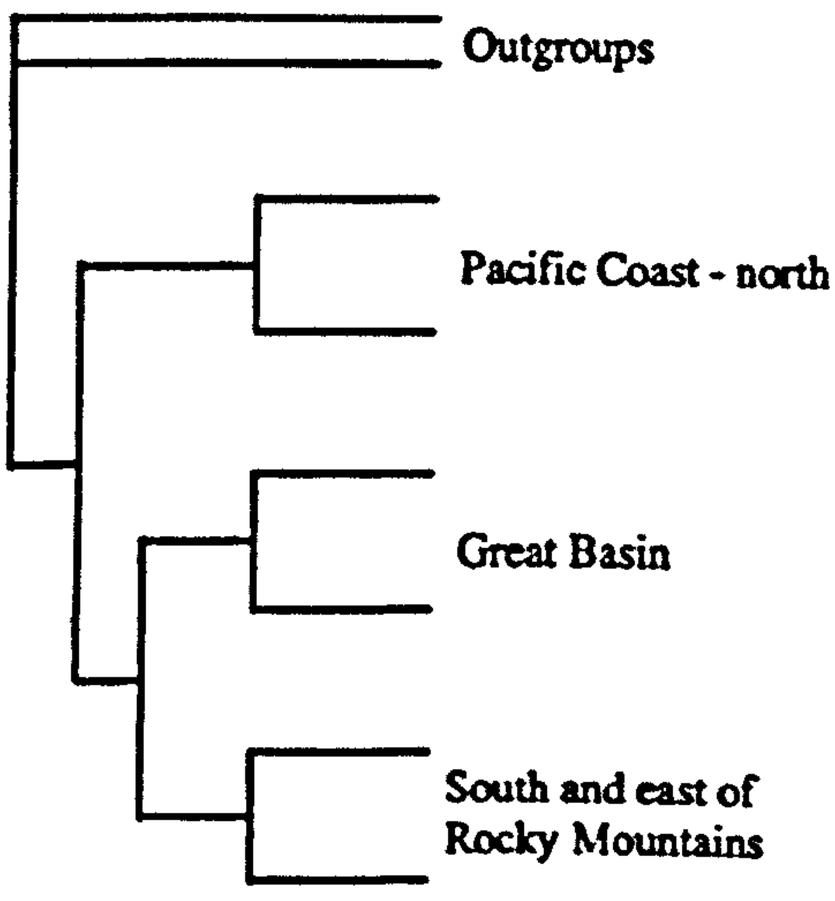
((*ab*,*ca*,*co*,*ce*,*he*,*lu*,*or*,*vi*,(*co*,*nu*)),*C. durissus*, *C. scutulatus*)



Generalised *Crotalus viridis*
constraint phylogeny
(outgroup(SER(AZ,((PC-N,GB),(PC-S)))))



Generalised *Pituophis catenifer*
constraint phylogeny
(outgroup((SER,AZ),(PC-N),(GB,PC-S))))



Generalised *Phrynosoma*
constraint phylogeny
(outgroup(PC-N(GB),(SER)))

Appendix IV. Museum specimens

Specimens are ordered alphabetically by state, then by county. Abbreviations are as follows:

Museum acronyms - **BM**, British Museum of Natural History; **CAS** or **CAS-SU**, California Academy of Sciences; **CMNH**, Carnegie Museum of Natural History; **FMNH**, Field Museum of Natural History, Chicago; **UMKC**, Museum of Natural History, University of Kansas; **LACM**, Los Angeles County Museum; **MVZ**, Museum of Vertebrate Zoology, Berkeley; **TCWC**, Texas Cooperative Wildlife Collection, Texas A & M University; **UMMZ**, University Michigan Museum of Zoology; **UMNH**, Utah Museum of Natural History; **USNM**, United States National Museum (The Smithsonian Institute); **UTEP**, University of Texas, El Paso; **Z**, San Diego Natural History Museum.

U. S. States - **AZ**, Arizona; **CA**, California; **CO**, Colorado; **ID**, Idaho; **KS**, Kansas; **MT**, Montana; **NV**, Nevada; **NM**, New Mexico; **OK**, Oklahoma; **OR**, Oregon; SD, South Dakota; **TX**, Texas; **UT**, Utah; **WA**, Washington; **WY**, Wyoming; *Mexico*, **MX**; *Canada*, **CD**.

Conventional subspecies of C. viridis - ***ab***, *abyssus*; ***ca***, *caliginis*; ***ce***, *cerberus*; ***co***, *concolor*; ***he***, *helleri*; ***lu***, *lutosus*; ***nu***, *nuntius*; ***or***, *oreganus*; ***vi***, *viridis*. Sex: (m) male; (f) female. Specimens also included in both the venom study (Chapter 5) and the molecular phylogeny (Chapter 2) are underscored and asterisked. Specimens also included in the molecular phylogeny, but not the venom study, are underscored. One exception, CAS-RL001 was included in the venom study, but not the phylogeny.

Numbers in parentheses are latitude and longitude coordinates for each locality.

CAS 192803 (f) - Hwy 666, Apache Co., AZ [nu] (34.250109.375); **CAS 192808** (f) - Eager, Apache Co., AZ [nu] (34.133109.285); **CAS 63899** (f) - Bibo, Apache Co., AZ [nu] (35.200109.720); **CAS 170511** (m) - Hwy 63 where it crosses 64, Apache Co., AZ [nu] (36.875109.050); **CAS 192795** (m) - Hwy 666, Apache Co., AZ [nu] (34.250, 109.375); **CAS 192796** (m) - Hwy 666, Apache Co., AZ [nu] (34.250, 109.375); **CAS 192806** (m) - Hwy 666, Apache Co., AZ [nu] (34.250, 109.375); **CAS 63900** (m) - Bibo, Apache Co., AZ [nu] (35.200, 109.720); **CAS-SU 10820** (m) - Adamana, Apache Co., AZ [nu] (34.977, 109.822); **MVZ 09330** (m) - Chas L. Camp, 40 mi S of Navajo, Apache Co., AZ [nu] (34.506, 109.360); **MVZ 56980** (m) - Hunt, Apache Co., AZ [nu] (34.610, 109.627); **MVZ 9329** (m) - Chas L. Camp, 40 mi S of Navajo, Apache Co., AZ [nu] (34.506, 109.360); **CAS 170509** (f) - Hwy 99, Coconino Co., AZ [nu] (34.700, 110.780); **CAS 63898** (f) - Angell, Coconino Co., AZ [nu] (35.196, 111.303); **CAS 63902** (f) - Sunshine, Coconino Co., AZ [nu] (35.124, 111.031); **CAS 63905** (f) - Sunshine, Coconino Co., AZ [nu] (35.124, 111.031); **CAS 63906** (f) - Grand Canyon, Coconino Co., AZ [ab] (36.063, 112.242); **LACM 28043** (f) - Flagstaff, Coconino Co., AZ [ce] (35.198, 111.651); **MVZ 18051** (f) - Dripping Springs, S rim, Grand Canyon, Coconino Co., AZ [nu] (36.063, 112.242); **MVZ 8683** (f) - Deadmans Flat, San Francisco Mountain, Coconino Co., AZ [nu] (35.460, 111.563); **UMMZ 205057** (f) - Meteor Crater, Coconino Co., AZ [nu] (35.028, 111.023); **UMMZ 59834** (f) - Canon Padre, Coconino Co., AZ [nu] (35.217, 111.108); **USNM 239262** (f) - Specific locality unknown, Coconino Co., AZ [ab] (35.196, 111.303); **Z 02272** (f) - Grand Canyon Trail, South Rim, Grand Canyon, Coconino Co., AZ [ab] (36.063, 112.242); **Z 03254** (f) - Desert View Point (Navajo Point), Grand Canyon, Coconino Co., AZ [ab] (36.042, 111.824); **Z 09524** (f) - Indian Gardens, Grand Canyon, Coconino Co., AZ [ab] (36.079, 112.120); **Z 1495** (f) - Winslow, Coconino Co., AZ [nu] (35.024, 110.697); **Z 3529** (f) - Sunshine, Coconino Co., AZ [nu] (35.124, 111.031); **CAS 156187** (m) - West Bacobi, Coconino Co., AZ [nu] (35.925, 110.656); **CAS 170503** (m) - Page, Coconino Co., AZ [nu] (36.909, 111.473); **CAS 192809** (m) - Hwy 89, Wupatki National Monument Road, Coconino Co., AZ [nu] (35.558, 111.353); **CAS 63897** (m) - Angell, Coconino Co., AZ [nu] (35.196, 111.303); **CAS 63901** (m) - Sunshine, Coconino Co., AZ [nu] (35.124, 111.031); **CAS 63903** (m) - Sunshine, Coconino Co., AZ [nu] (35.124, 111.031); **CAS 63904** (m) - Sunshine, Coconino Co., AZ [nu] (35.124, 111.031); **CMNH 20294** (m) - Bright Angel Creek, North Rim Grand

Canyon, Coconino Co., AZ [ab] (36.099, 112.092); **LACM 105112** (m) - Tuba City, Coconino Co., AZ [nu] (36.135, 111.239); **LACM 105114** (m) - Tuba City, Coconino Co., AZ [nu] (36.135, 111.239); **LACM 3120** (m) - Muav Saddle, Coconino Co., AZ [lu] (36.332, 112.357); **LACM 52595** (m) - Tuba City, Coconino Co., AZ [nu] (36.135, 111.239); **LACM 52596** (m) - Tuba City, Coconino Co., AZ [nu] (36.135, 111.239); **LACM 52597** (m) - Tuba City, Coconino Co., AZ [nu] (36.135, 111.239); **MVZ 16030** (m) - Tanners Tank, Coconino Co., AZ [nu] (35.694, 111.550); **MVZ 77079** (m) - US route 89 6 mi SE of Colorado River, Coconino Co., AZ [nu] (36.870, 111.500); **MVZ 8684** (m) - Deadman's Flat, Coconino Co., AZ [nu] (35.460, 111.563); **MVZ 8685** (m) - Deadman's Flat, San Francisco Mountain, Coconino Co., AZ [nu] (35.460, 111.563); **MVZ 8942** (m) - Chas L. Camp, 40 mi S of Navajo, Coconino Co., AZ [ce] (34.506, 109.360); **MVZ 8943** (m) - Chas L. Camp, 40 mi S of Navajo, Coconino Co., AZ [nu] (34.506, 109.360); UCWB Rimmy Jim (m) - Rimmy Jim, Coconino Co., AZ [nu] (35.716, 111.472)*; **UMMZ 189784** (m) - Meteor Crater, Coconino Co., AZ [nu] (35.028, 111.023); **USNM 239361** (m) - Cardenas, Grand Canyon, Coconino Co., AZ [ab] (36.087, 111.843); **USNM 310836** (m) - Cave Springs, Kaibab, Grand Canyon, Coconino Co., AZ [ab] (36.519, 110.970); **Z 02600** (m) - Kaibab Trail, Grand Canyon, Coconino Co., AZ [ab] (36.169, 112.039); **Z 02657** (m) - Indian Gardens, Grand Canyon, Coconino Co., AZ [ab] (36.079, 112.120); **Z 25794** (m) - Indian Gardens, Grand Canyon, Coconino Co., AZ [ab] (36.079, 112.120); **Z 25795** (m) - Indian Gardens, Grand Canyon, Coconino Co., AZ [ab] (36.079, 112.120); **Z 3737** (m) - Sunshine, Coconino Co., AZ [nu] (35.124, 111.031); **Z 3738** (m) - Sunshine, Coconino Co., AZ [nu] (35.124, 111.031); **Z 3739** (m) - Sunshine, Coconino Co., AZ [nu] (35.124, 111.031); **CAS 192802** (f) - Sierra Ancha, Gila Co., AZ [ce] (33.958, 111.186); **CMNH 51914** (f) - Parker Creek, Gila Co., AZ [ce] (33.723, 111.010); **CMNH 51915** (f) - Asbestos Peak, Wedge, Gila Co., AZ [ce] (33.394, 110.786); **UMKC 49667** (f) - Globe, Gila Co., AZ [nu] (33.394, 110.786); **UMMZ 134043** (f) - Pine, Gila Co., AZ [ce] (34.384, 111.454); **UMMZ134042** (f) - Payson, Gila Co., AZ [ce] (34.231, 111.324); **CMNH 51382** (m) - Payson, Gila Co., AZ [ce] (34.231, 111.324); **CMNH 51521** (m) - Sierra Ancha Exp. Forest, Gila Co., AZ [ce] (33.958, 111.186); **CMNH 51833** (m) - ?, Gila Co., AZ [ce] (33.394, 110.786); **CMNH 51834** (m) - ?, Gila Co., AZ [ce] (33.394, 110.786); **CMNH 51835** (m) - ?, Gila Co., AZ [ce] (33.394, 110.786); **UMKC 173099** (m) - Payson, Gila Co., AZ [ce] (34.231, 111.324); **MVZ 6311** (m) - Sierra Ancha, Gila Co., AZ [ce] (33.958, 111.186); **TCWC 21913** (m) - Salt River Canyon, Gila Co., AZ [ce] (33.619, 110.922); **USNM 105242** (m) - Roosevelt Reservoir, Parker Creek, Gila Co., AZ [ce] (33.723, 111.010); **USNM 105243** (m) - Roosevelt Reservoir, Parker Creek, Gila Co., AZ [ce] (33.723, 111.010); **CAS 170508** (f) - 5.5 mi NE Jct Aravapai Rd and Klondyke Rd, Graham Co., AZ [ce] (32.500, 110.330); **CMNH 26708** (f) - San Carlos, Graham Co., AZ [ce] (33.345, 110.453); **CAS 170518** (m) - hwy 366 at entrance to National Forest, Graham Co., AZ [ce] (32.690, 109.800); **CMNH 26674** (m) - San Carlos, Graham Co., AZ [ce] (33.345, 110.453); **CMNH 48740** (m) - Marijilda Canyon, Graham Co., AZ [ce] (32.741, 109.711); **CMNH 70922** (m) - Jafford, Graham Co., AZ [ce] (32.834, 109.707); **CMNH 70929** (m) - Marijilda Canyon, Graham Co., AZ [ce] (32.741, 109.711); **CMNH 70951** (m) - Marijilda Canyon, Graham Co., AZ [ce] (32.741, 109.711); **CMNH 70975** (m) - Jafford, Graham Co., AZ [ce] (32.834, 109.707); UCWB Uncle cerberus (m) - Stockton Pass Wash, Pinaleno Mts, Graham Co., AZ [ce] (32.649, 109.725)*; **CMNH 71095** (m) - Eagle Creek, Hot Springs Canyon, Greenlee Co., AZ [ce] (32.960, 109.406); **CAS 17539** (m) - Cave Creek, MaricopaAZ [ce] (33.567, 112.108); **USNM 161167** (f) - Sunflower, Maricopa Co., AZ [ce] (33.864, 111.467); **CMNH 47741** (m) - Sunflower, Maricopa Co., AZ [ce] (33.864, 111.467); **Z 33045** (f) - Pipe Springs Natl. Monument, Mohave Co., AZ [ab] (36.862, 112.736); **CAS 170507** (m) - Stone Lodge recreation area, Hualapai Mountain Park, Mohave Co., AZ [ce] (35.089, 113.898); **CAS 170517** (m) - Hualapai Mountains, Wabayma Park, Mohave Co., AZ [ce] (35.089, 113.898); **CAS 63907** (f) - Winslow, Navajo Co., AZ [nu] (35.024, 110.697); **CAS 170444** (m) - Holbrook, Navajo Co., AZ [nu] (34.902, 110.158); **CAS 192798** (m) - Seba Dalhai, Navajo Co., AZ [nu] (35.509, 110.448); **CAS 192798** (m) - Seba Dalhai, Hwy 87, Navajo Co., AZ [nu] (35.509, 110.448); **LACM 121579** (m) - White Cone Zone TP, Navajo Co., AZ [nu] (35.605, 110.068); **Z 1495** (m) - Winslow, Navajo Co., AZ [nu] (35.024, 110.697); **CAS 179421** (f) - 42.3 mi N of I10, Mescal Rd, hwy 297, Pima Co., AZ [ce] (32.153, 110.466); **CAS 170504** (m) - 16.2 mi N of I10, Mescal Rd, exit 297, Pima Co., AZ [ce] (32.153, 110.466); **CAS 170510** (m) - 16.2 mi N of I10, Mescal Rd, exit 297, Pima Co., AZ [ce] (32.153, 110.466); **CAS 34683** (m) - Mt Lemmon, Santa Catalina Mountains, Pima Co., AZ [ce] (32.443, 110.788); **UMKC 6635** (m) - Mount Lemmon, Pima Co., AZ [ce] (32.443, 110.788); **UMKC 19755** (f) - Specific locality unknown, Yavapai Co., AZ [ce] (34.540, 112.468); **CAS 35237** (m) - Oak Creek, Flagstaff, Yavapai Co., AZ [ce] (34.779, 111.763); **UMKC 19753** (m) - ?, Yavapai Co., AZ [ce] (34.540, 112.468); **UMKC 19754** (m) - ?, Yavapai Co., AZ [ce] (34.540, 112.468); **UMKC 23080** (m) - Prescott, Yavapai Co., AZ [ce] (34.540, 112.468); **UMMZ 206541** (m) - Red Rock State Park, Yavapai Co., AZ [ce] (34.825, 111.805); CAS RL001 (f) - Corrall Hollow, Alameda Co., CA [or] (37.656, 121.486)*; **CMNH 69881** (f) - Berkeley, Alameda Co., CA [or] (37.872, 122.272); **CAS 205965** (m) - French Creek, east side Plumas Natl. Forest, Butte Co., CA [or] (39.618, 121.094); **CAS 43559** (f) - Antioch, Contra Costa Co., CA [or] (38.005, 121.805); **UMKC 5329** (f) - Lake Tahoe, El Dorado Co., CA [or] (39.083, 120.033); **CAS 208784** (f) - ?, Fresno Co., CA [or] (36.460, 120.700); **CAS 208785** (f) - ?, Fresno Co., CA [or] (36.460, 120.700); **CAS-SU 23215** (f) - Caolinga, Fresno Co., CA [or] (36.140, 120.359); **CAS 170522** (m) - Sycamore Creek, E of Fresno, Fresno Co., CA [or] (36.920, 119.277); **CAS 191129** (m) - Panoche Road, nr I5, Fresno Co., CA [or] (36.460, 120.700); **CAS 208817** (m) - ?, Fresno Co., CA [or] (36.460, 120.700); **CAS-SU 10763** (f) - Scotia, Humboldt Co., CA [or] (40.483, 124.100); **CAS-SU 10766** (f) - Scotia, Humboldt Co., CA [or] (40.483, 124.100); **CAS-SU 10761** (m) -

Scotia, Humboldt Co., CA [or] (40.483, 124.100); **CAS 180329** (f) - Cedar Creek, Kern Co., CA [or] (35.642, 118.741); **CAS 192646** (f) - McKittick, Kern Co., CA [or] (35.306, 119.622); **CAS 38954** (m) - Tehachapi Mountains, Kern Co., CA [or] (35.081, 118.620); **CAS 38955** (m) - Live Oak Canyon, Tehachapi Mountains, Kern Co., CA [or] (35.081, 118.620); **CAS 38956** (m) - Live Oak Canyon, Tehachapie Mountains, Kern Co., CA [or] (35.081, 118.620); **CAS 43426** (m) - Santos Creek, Kern Co., CA [or] (35.489, 119.750); **CAS 203605** (f) - Cleaarlake Oaks, Lake Co., CA [or] (39.026, 122.671); **BM 1996.157** (m) - Madeline, Lassen Co., CA [lu] (41.051, 120.474); **CAS 44220** (m) - Eagle Lake, Lassen Co., CA [lu] (40.645, 120.743); **CAS 44221** (m) - Eagle Lake, Lassen Co., CA [lu] (40.645, 120.743); **LACM 105147** (f) - Wrightwood, Inspiration Point, Los Angeles Co., CA [he] (36.361, 117.633); **LACM 20066** (f) - Franklin Canyon, Los Angeles Co., CA [he] (34.103, 118.412); **LACM 105134** (m) - Western, Los Angeles Co., CA [he] (34.178, 118.282); **LACM 105136** (m) - San Pedro, la Vista Verde, Los Angeles Co., CA [he] (33.736, 118.291); **LACM 20065** (m) - Franklin Canyon, Santa Monica Mountains, Los Angeles Co., CA [he] (34.103, 118.412); **CAS 192629** (f) - Raymond, Madera Co., CA [or] (37.210, 119.904); **CAS 192630** (m) - Raymond, Madera Co., CA [or] (37.210, 119.904); **BM 1988.1037** (f) - Covelo, Mendocino Co., CA [or] (39.793, 123.247); **CAS 10901** (m) - ?, Mendocino Co., CA [or] (39.793, 123.247); **CAS 13639** (m) - Los Banos, Merced Co., CA [or] (37.058, 120.849); **CAS 192637** (m) - Clear Lake Dam, Modoc Co., CA [lu] (41.927, 121.075); **MVZ 2079** (m) - Parker Creek, Warner Mountains, Modoc Co., CA [lu] (41.522, 120.476); **MVZ 16341** (m) - Levering, Mono Co., CA [lu] (38.893, 119.179); **MVZ 17619** (m) - East Walker River, John Murphy Creek, Mono Co., CA [lu] (38.893, 119.179); **Z 35835** (f) - Monterey, Monterey Co., CA [or] (36.600, 121.894); **Z 37370** (f) - Monterey, Monterey Co., CA [or] (36.600, 121.894); **Z 27535** (m) - Nacimiento, Monterey Co., CA [or] (35.809, 120.741); **Z 35347** (m) - Monterey, Monterey Co., CA [or] (36.600, 121.894); **Z 36228** (m) - Monterey, Monterey Co., CA [or] (36.600, 121.894); **CAS 206278** (f) - Slate Creek, Plumas Natl. Forest, Plumas Co., CA [or] (39.943, 121.016); **CAS 206279** (f) - Slate Creek, Plumas Natl. Forest, Plumas Co., CA [or] (39.943, 121.016); **CAS 206280** (m) - Slate Creek, Plumas Co., CA [or] (39.943, 121.016); **CAS-SU 1804** (f) - San Jacinto, Riverside Co., CA [he] (33.784, 116.958); **CAS 200713** (m) - Metropolitan Water District, Riverside Co., CA [he] (33.918, 116.786); **CMNH 91581** (m) - Cabazon, Riverside Co., CA [he] (33.918, 116.786); **CAS 192613** (m) - Paicines, San Benito Co., CA [or] (36.510, 121.081); **CAS 192625** (m) - Paicines, San Benito Co., CA [or] (36.510, 121.081); **CAS 91630** (m) - Pinnacles Natl Monument, San Benito Co., CA [or] *(36.510, 121.081)*; **CAS-SU 23216** (m) - Panoche Valley, San Benito Co., CA [or] (36.510, 121.081); **LACM** *105166* (f) - Devore, San Bernardino Co., CA [he] *(34.216, 117.401)*; **LACM** *105168* (f) - Cedar Springs, San Bernardino Co., CA [he] (34.283, 117.333); **LACM** *105173* (f) - *Lower Covington Flats Road, San Bernardino Co., CA [he] (34.038, 116.308)*; **CAS 170498** (m) - Puddington Reservoir, San Bernardino Co., CA [he] *(34.503, 116.921)*; **LACM** *105169* (m) - Cedar Springs, San Bernardino Co., CA [he] (34.283, 117.333); **LACM** *105170* (m) - Cedar Springs, San Bernardino Co., CA [he] (34.283, 117.333); **LACM** *105174* (m) - Lucerne Valley, 21mi SE Daggett, San Bernardino Co., CA [he] (34.503, 116.921); **CAS 170413** (f) - San Diego Zoo, San Diego Co., CA [he] (32.715, 117.156); **CAS 170418** (f) - San Diego Zoo, San Diego Co., CA [he] (32.715, 117.156); **CAS 170422** (f) - San Diego Zoo, San Diego Co., CA [he] (32.715, 117.156); **UMKC 8477** (f) - La Jolla, San Diego Co., CA [he] (32.847, 117.273); **Z 42201** (f) - Escondido, San Diego Co., CA [he] (33.119, 117.086); **CAS 170414** (m) - San Diego Zoo, San Diego Co., CA [he] (32.715, 117.156); **CAS 170424** (m) - ?, San Diego Co., CA [he] (32.606, 116.468); **CAS 170425** (m) - San Diego Zoo, San Diego Co., CA [he] (32.715, 117.156); **CAS 40088** (m) - Warrens Ranch, San Diego Co., CA [he] (32.999, 116.998); **CAS 57860** (m) - Campo, San Diego Co., CA [he] (32.606, 116.468); **UMKC 5328** (m) - Pelomar Mountains, San Diego Co., CA [he] (32.847, 117.273); **CAS 22971** (f) - Templeton, San Luis Obispo Co., CA [or] (35.550, 120.705); **CAS 43420** (f) - Indian Creek, San Luis Obispo Co., CA [or] (35.654, 120.450); **UMKC 33453** (f) - Sly Creek, San Luis Obispo Co., CA [he] (35.351, 119.986); **Z 25442** (f) - Morro Rock, San Luis Obispo Co., CA [or] (35.369, 120.867); **Z 27520** (f) - Shandon, San Luis Obispo Co., CA [or] (35.655, 120.374); **Z 37453** (f) - Shandon, San Luis Obispo Co., CA [or] (35.655, 120.374); **Z 39945** (f) - Paso Robles, San Luis Obispo Co., CA [or] (35.627, 120.690); **CAS 192683** (m) - Simmler, San Luis Obispo Co., CA [or] (35.351, 119.986); **CAS 197570** (m) - Soda Lake, San Luis Obispo Co., CA [he] (35.235, 119.892); **Z 37450** (m) - Shandon, San Luis Obispo Co., CA [or] (35.655, 120.374); **Z 37451** (m) - Shandon, San Luis Obispo Co., CA [or] (35.655, 120.374); **Z 37452** (m) - Shandon, San Luis Obispo Co., CA [or] (35.655, 120.374); **Z 37454** (m) - Shandon, San Luis Obispo Co., CA [or] (35.655, 120.374); **CAS-SU 19871** (f) - Belmont, San Mateo Co., CA [or] (37.520, 122.275); **CAS-SU 19872** (m) - Belmont, San Mateo Co., CA [or] (37.520, 122.275); **CAS-SU 8099** (m) - Stone circle, Woodside Road, San Mateo Co., CA [or] (37.430, 122.253); **UMMZ 79342** (f) - Maricopa, Santa Barbara Co., CA [or] (35.059, 119.400); **UMMZ 79345** (f) - Maricopa, Santa Barbara Co., CA [or] (35.059, 119.400); **Z 32134** (f) - Santa Barbara, Santa Barbara Co., CA [he] (34.421, 119.697); **Z 58234** (f) - Santa Barbara, Santa Barbara Co., CA [he] (34.421, 119.697); **CMNH 54019** (m) - Toro Canyon, Santa Barbara Co., CA [or] (34.429, 119.575); **CMNH 54017** (m) - Toro Canyon, nr Santa Barabara, Santa Barbara Co., CA [or] (34.429, 119.575); **UMMZ 79341** (m) - Maricopa, Santa Barbara Co., CA [or] (35.059, 119.400); **UMMZ 79343** (m) - Maricopa, Santa Barbara Co., CA [or] (35.059, 119.400); **UMMZ 79344** (m) - Maricopa, Santa Barbara Co., CA [or] (35.059, 119.400); **Z 13715** (m) - Santa Barbara, Santa Barbara Co., CA [he] (34.421, 119.697); **CAS 192672** (f) - Anderson Reservoir, Santa Clara Co., CA [or] (37.166, 121.628); **CAS 78697** (f) - Alma, Santa Clara Co., CA [or] (37.182, 122.001); **CAS 192635** (m) - Alum Rock Park, Santa Clara Co., CA [or] (37.398, 121.799); **CAS 192661** (m) - Smith Creek, Santa Clara Co., CA [or] (37.383, 121.692); **CAS 192670** (m) - Mount Unynhum, Santa

Clara Co., CA [or] (37.383, 121.692); **CAS 192674** (m) - Hwy 30 between Halls Valley and Smith Creek, Santa Clara Co., CA [or] (37.319, 121.699); **CAS-SU 10024** (m) - La Honda, Santa Clara Co., CA [or] (37.319, 122.273); **CAS 192669** (f) - Saratoga Gap, Santa Cruz Co., CA [or] (37.258, 122.120); **CAS 71332** (m) - Glenbrook, Santa Cruz Co., CA [or] (37.383, 121.692); **CAS 36064** (f) - Gazelle, Siskiyou Co., CA [or] (41.521, 122.519); **CAS-SU 7690** (f) - Klamath Hot Springs, Beswick, Siskiyou Co., CA [or] (41.973, 122.204); **MVZ 16439** (f) - Hornbrook, Siskiyou Co., CA [or] (41.910, 122.555); **MVZ 18405** (f) - Klamath River, Siskiyou Co., CA [or] (41.861, 122.824); **MVZ 18408** (f) - Clear Creek, 3 mi w Klamath River, Siskiyou Co., CA [or] (41.376, 122.140); **MVZ 18404** (m) - Clear Creek, 3 mi west of Klamath River, Siskiyou Co., CA [or] (41.376, 122.140); **MVZ 18407** (m) - Clear Creek, 3 mi west of Klamath River, Siskiyou Co., CA [or] (41.376, 122.140); **CAS 78707** (f) - Sonoma Mountains, Sonoma Co., CA [or] (38.300, 122.550); **CAS 78706** (m) - Sonoma Mountains, Sonoma Co., CA [or] (38.300, 122.550); **CAS 78795** (m) - Sonoma Mountains, Sonoma Co., CA [or] (38.300, 122.550); **CAS 80919** (m) - Kenwood, Sonoma Co., CA [or] (38.414, 122.545); **CAS 161979** (f) - Del Puerto Canyon Road 10 mi w Hwy5, Stanislaus Co., CA [or] (37.488, 121.206); **CAS 192643** (f) - Raines Park, Stanislaus Co., CA [or] (37.421, 121.371); **CAS 78696** (m) - Carville Mining District, Trinity Co., CA [or] (37.421, 121.371); **CMNH 69526** (f) - Three Rivers, Sequoia Natl Park, Tulare Co., CA [or] (36.439, 118.904); **CAS-SU 5233** (m) - Delano, Tulare Co., CA [or] (35.769, 119.246); **CAS-SU 8890** (m) - Sequoia Natl. Park, Cave Creek, Tulare Co., CA [or] (36.585, 118.832); **CAS-SU 8924** (m) - Sequoia Natl Park, Sunset Rock, Tulare Co., CA [or] (36.577, 118.775); **CAS-SU 10902** (m) - Cascade Creek Public camp ground, Tuolumne Co., CA [or] (38.280, 119.969); **CMNH 34868** (f) - Ojai, Ventura Co., CA [or] (34.448, 119.242); **CMNH 49026** (m) - Ventura, Ventura Co., CA [he] (34.278, 119.292); **BM 63.8.20.20** (f) - Pateros, British Columbia, Canada [or] (48.051, 119.902); **Z 36407** (f) - Vernon, British Columbia, Canada [or] (55.267, 119.267); **Z 36409** (f) - Vernon, British Columbia, Canada [or] (55.267, 119.267); **Z 36410** (f) - Vernon, British Columbia, Canada [or] (55.267, 119.267); **CAS 170489** (f) - Walsh, Baca Co., CO [vi] (37.386, 102.278); **CAS 179487** (f) - Walsh, Baca Co., CO [vi] (37.386, 102.278); **CAS 170497** (m) - Blanca, Costilla Co., CO [vi] (37.438, 105.515); **CAS 100836** (m) - Colorado Springs, El Paso Co., CO [vi] (38.834, 104.821); **CAS 100839** (m) - Colorado Springs, El Paso Co., CO [vi] (38.834, 104.821); **CMNH 39251** (f) - Golden, Jefferson Co., CO [vi] (39.756, 105.221); **CAS 170455** (f) - Craig, Moffat Co., CO [vi] (40.515, 107.546); **CAS 170457** (f) - Slader, Moffat Co., CO [vi] (40.515, 107.546); **CAS 170458** (f) - Slader, Moffat Co., CO [vi] (40.515, 107.546); **UMKC 32507** (f) - Craig, Moffat Co., CO [vi] (40.515, 107.546); **UMKC 32508** (f) - Craig, Moffat Co., CO [vi] (40.515, 107.546); **CAS 179456** (m) - Slader, Moffat Co., CO [vi] (40.515, 107.546); **CMNH 21969** (m) - Sand Wash, 20 mi N of Maybell, Moffat Co., CO [vi] (40.515, 107.546); **UMKC 32509** (m) - Craig, Moffat Co., CO [vi] (40.515, 107.546); **UMKC 32510** (m) - Craig, Moffat Co., CO [vi] (40.515, 107.546); **UMKC 32511** (m) - Craig, Moffat Co., CO [vi] (40.515, 107.546); **CMNH 48892** (f) - 3.2m W of Two Buttes Mountain, Prowers Co., CO [vi] (37.659, 102.542); **CAS 170446** (f) - Greenley, Weld Co., CO [vi] (40.215, 104.822); **CAS 179443** (f) - Greeley, Weld Co., CO [vi] (40.423, 104.709); **CAS 65095** (f) - Platteville, Weld Co., CO [vi] (40.215, 104.822); **CAS 65103** (f) - Platteville, Weld Co., CO [vi] (40.215, 104.822); **CAS 100835** (m) - Colorado Springs, Weld Co., CO [vi] (38.834, 104.821); **CAS 65106** (m) - Platteville, Weld Co., CO [vi] (40.215, 104.822); **CAS 65108** (m) - Platteville, Weld Co., CO [vi] (40.215, 104.822); **CAS 64120** (f) - Boise, Ada Co., ID [lu] (43.614, 116.203); **CAS 64121** (f) - Boise, Ada Co., ID [lu] (43.614, 116.203); **CAS 64127** (f) - Boise, Ada Co., ID [lu] (43.614, 116.203); **CAS 64195** (f) - Boise, Ada Co., ID [lu] (43.614, 116.203); **FMNH 6569** (f) - ?, Ada Co., ID [lu] (43.456, 116.136); **CAS 64122** (m) - Boise, Ada Co., ID [lu] (43.614, 116.203); **CAS 64191** (m) - Snake River, Swan Falls, Ada Co., ID [lu] (43.242, 116.373); **CAS 64192** (m) - Snake River, Swan Falls, Ada Co., ID [lu] (43.242, 116.373); **CAS 64193** (m) - Snake River, Swan Falls, Ada Co., ID [lu] (43.242, 116.373); **CAS 64194** (m) - Boise, Ada Co., ID [lu] (43.614, 116.203); **CAS 64196** (m) - Boise, Ada Co., ID [lu] (43.614, 116.203); **CAS 64208** (m) - Mt Kome, Ada Co., ID [lu] (43.614, 116.203); **FMNH 6961** (m) - Blacks Creek Canyon, Ada Co., ID [lu] (43.456, 116.136); **FMNH 6962** (m) - Blacks Creek Canyon, Ada Co., ID [lu] (43.456, 116.136); **Z 8476** (m) - Council, Adams Co., ID [lu] (44.729, 116.438); **Z 8477** (m) - Council, Adams Co., ID [lu] (44.729, 116.438); **Z 8479** (m) - Council, Adams Co., ID [lu] (44.729, 116.438); **Z 8480** (m) - Council, Adams Co., ID [lu] (44.729, 116.438); **Z 8481** (m) - Council, Adams Co., ID [lu] (44.729, 116.438); **Z 8482** (m) - Council, Adams Co., ID [lu] (44.729, 116.438); **Z 8483** (m) - Council, Adams Co., ID [lu] (44.729, 116.438); **Z 8484** (m) - Council, Adams Co., ID [lu] (44.729, 116.438); **Z 8485** (m) - Council, Adams Co., ID [lu] (44.729, 116.438); **Z 8486** (m) - Council, Adams Co., ID [lu] (44.729, 116.438); **CAS 55225** (f) - Melba, Canyon Co., ID [lu] (43.376, 116.528); **CAS 55226** (f) - Melba, Canyon Co., ID [lu] (43.376, 116.528); **CAS 55227** (f) - Melba, Canyon Co., ID [lu] (43.376, 116.528); **CAS 55223** (f) - Cleft, Elmore Co., ID [lu] (43.239, 115.850); **CAS 55224** (f) - Cleft, Elmore Co., ID [lu] (43.239, 115.850); **CAS 55233** (m) - Cleft, Elmore Co., ID [lu] (43.239, 115.850); **UTEP 8452** (m) - Julialetta, LatahID [or] (46.579, 116.705); **CAS 170490** (f) - Jctn 12, hwy 12, Nez Perce Co., ID [or] (46.340, 117.000); **CAS 170491** (m) - Jctn 12, hwy 12, Nez Perce Co., ID [or] (46.340, 117.000); **CAS 170492** (m) - ?, Nez Perce Co., ID [or] (46.340, 117.000); **CAS 170493** (m) - ?, Nez Perce Co., ID [or] (46.340, 117.000); **CAS 172071** (m) - Hwy 12 10.7 mi E jct 12, Nez Perce Co., ID [or] (46.340, 117.000); **UTEP 8451** (m) - Cambridge, 8.3 mi W, Washington Co., ID [lu] (44.573, 116.675); **BM 1980.1149** (f) - State Lake, Clark Co., KS [vi] (37.383, 99.782); **CMNH 8649** (m) - Moore Springs, Ford Co., KS [vi] (37.383, 99.782); **CAS 12141** (f) - San Quintin, Baja California, Mexico [he] (30.005, 108.783); **CAS 182512** (f) - San Quintin, Baja California, Mexico [he] (30.005, 108.783); **CAS 56863** (f) - La Encantada, Baja California, Mexico [he] (31.000, 115.400); **CAS 12154** (m) - ?, Baja

California, Mexico [he] (31.000, 115.400); **CAS 56864** (m) - La Encantada, Baja California, Mexico [he] (31.000, 115.400); **CAS 56882** (m) - Valladores Creek, Baja California, Mexico [he] (31.750, 115.667); **CAS 56883** (m) - Valladores Creek, Baja California, Mexico [he] (31.750, 115.667); **CAS 57555** (m) - San Antonio Del Mar, Baja California, Mexico [he] (31.083, 116.267); **CAS 85130** (m) - Sierra Juarez, Baja California, Mexico [he] (31.750, 115.667); **USNM 225376** (m) - Ojos Negros, Baja California, Mexico [he] (31.867, 116.267); **CAS 13583** (f) - Isla Coronado Sur, Los Coronados, Mexico [ca] (32.417, 117.250); **CAS 13584** (f) - Isla Coronado Sur, Los Coronados, Mexico [ca] (32.417, 117.250); **CAS 13603** (f) - Isla Coronado Sur, Los Coronados, Mexico [ca] (32.417, 117.250); **CAS 192684** (f) - Isla Coronado Sur, Los Coronados, Mexico [ca] (32.417, 117.250); **CAS 192686** (f) - Isla Coronado Sur, Los Coronados, Mexico [ca] (32.417, 117.250); **LACM 20140** (f) - Isla Coronado Sur, Los Coronados, Mexico [ca] (32.417, 117.250); **LACM 20141** (f) - Isla Coronado Sur, Los Coronados, Mexico [ca] (32.417, 117.250); **LACM 20145** (f) - Isla Coronado Sur, Los Coronados, Mexico [ca] (32.417, 117.250); **Z 13714** (f) - Isla Coronado Sur, Los Coronados, Mexico [ca] (32.417, 117.250); **Z 13715** (f) - Isla Coronado Sur, Los Coronados, Mexico [ca] (32.417, 117.250); **BM 92.2.11.34** (m) - Isla Coronado Sur, Los Coronados, Mexico [ca] (32.417, 117.250); **UMKC 3559** (m) - Isla Coronado Sur, Los Coronados, Mexico [ca] (32.417, 117.250); **LACM 20139** (m) - Isla Coronado Sur, Los Coronados, Mexico [ca] (32.417, 117.250); **LACM 20143** (m) - Isla Coronado Sur, Los Coronados, Mexico [ca] (32.417, 117.250); **LACM 20144** (m) - Isla Coronado Sur, Los Coronados, Mexico [ca] (32.417, 117.250); **Z 13713** (m) - Isla Coronado Sur, Los Coronados, Mexico [ca] (32.417, 117.250); **Z 200700** (m) - Isla Coronado Sur, Los Coronados, Mexico [ca] (32.417, 117.250); **Z 20078** (m) - Isla Coronado Sur, Los Coronados, Mexico [ca] (32.417, 117.250); **UMKC 1620** (m) - Edgar, Carbon Co., MT [vi] (45.465, 108.852); **UMKC 130284** (m) - Carter, Carter Co., MT [vi] (47.781, 110.956); **CMNH 19985** (m) - Cascade, Cascade Co., MT [vi] (47.271, 111.700); **CMNH 23423** (m) - Cavern, Jefferson Co., MT [vi] (45.824, 111.856); **CMNH 30751** (m) - Pipestone Springs, Jefferson Co., MT [vi] (45.894, 112.230); **CAS 193217** (f) - Plains, Hwy 200, Sanders Co., MT [or] (47.460, 114.882); **CAS 192652** (m) - Quinns, Sanders Co., MT [vi] (47.325, 114.801); **CAS 192653** (m) - Plains, Hwy 200, Sanders Co., MT [vi] (47.460, 114.882); **UMKC 137618** (m) - Agency, Sanders Co., MT [vi] (47.328, 114.293); **CMNH S 6656** (f) - Kevin, Tooele Co., MT [vi] (48.745, 111.964); **S 6655** (f) - Kevin, Tooele Co., MT [vi] (48.745, 111.964); **CAS 170472** (f) - Harlowtown, Wheatland Co., MT [vi] (46.436, 109.834); **CAS 170494** (f) - Harlowtown, Wheatland Co., MT [vi] (46.436, 109.834); **CAS 170482** (m) - Harlowtown, Wheatland Co., MT [vi] (46.436, 109.834); **CAS 170483** (m) - Harlowtown, Wheatland Co., MT [vi] (46.436, 109.834); **FMNH 35030** (f) - Specific locality unknown, Cherry Co., NE [vi] (42.938, 100.955); **FMNH 35032** (f) - Specific locality unknown, Cherry Co., NE [vi] (42.938, 100.955); **FMNH 35031** (m) - ?, Cherry Co., NE [vi] (42.938, 100.955); **FMNH 35033** (m) - Specific locality unknown, Cherry Co., NE [vi] (42.938, 100.955); **FMNH 35814** (m) - ?, Cherry Co., NE [vi] (42.938, 100.955); **TCWC 63507** (f) - Norden Bridge, Keya Paha Co., NE [vi] (42.869, 100.074); **FMNH 26330** (m) - Bridgeport, Morrill Co., NE [vi] (41.665, 103.099); **CMNH 60602** (m) - West Mesa, Bernalillo Co., NM [vi] (35.085, 106.734); **CMNH 60603** (m) - West Mesa, Bernalillo Co., NM [vi] (35.085, 106.734); **CAS-SU 10886** (f) - Medley Tank, Datil, Catron Co., NM [vi] (34.171, 107.863); **CMNH 58959** (f) - Jornadoa Road, 5.6 mi N of US 70, Dona Ana Co., NM [vi] (31.856, 107.000); **TCWC 33386** (f) - Las Cruces, Dona Ana Co., NM [vi] (32.312, 106.778); **UTEP 12084** (f) - Fort Bliss Military Reserve, Dona Ana Co., NM [vi] (31.918, 108.320); **UTEP 17625** (f) - Hwy 9, 3.2 mi W of jctn with interstate rd, Dona Ana Co., NM [co] (37.871, 109.342)*; **UTEP 6818** (f) - 39mi E Columbus, border between Dona Ana and El Paso Counties, Dona Ana Co., NM [vi] (31.856, 107.000); **UTEP mm27** (f) - Hwy 9, nr. jctn with interstate rd, Dona Ana Co., NM [vi] (31.856, 106.639)*; **TCWC 33381** (m) - Las Cruces, Dona Ana Co., NM [vi] (32.312, 106.778); **TCWC 33383** (m) - Radium Springs, Dona Ana Co., NM [vi] (32.501, 106.928); **TCWC 33385** (m) - Las Cruces, Dona Ana Co., NM [vi] (32.312, 106.778); **TCWC 33387** (m) - Radium Springs, Dona Ana Co., NM [vi] (32.501, 106.928); **UTEP 17485** (m) - Santa Teresa, W Portillo Mountains, Dona Ana Co., NM [vi] (31.856, 106.639); **UTEP 17518** (m) - Specific locality unknown, Dona Ana Co., NM [vi] (31.856, 106.639); **UTEP mm26** (m) - Hwy 9, nr. jctn with interstate rd, Dona Ana Co., NM [vi] (32.501, 106.928)*; **TCWC 64435** (f) - Hatchita, Grant Co., NM [vi] (31.918, 108.320); **UTEP 17625** (f) - St Hwy 81 1.3 mi S jct with I Hwy 10, Grant Co., NM [vi] (31.856, 106.639); **UTEP 9986** (f) - US Hwy 81 10.2 mi from jct between 8 and 110, Grant Co., NM [vi] (31.918, 108.320); **TCWC 56337** (m) - Hachita, Grant Co., NM [vi] (31.918, 108.320); **UTEP 10604** (f) - Lordsburg, Hidalgo Co., NM [vi] (32.350, 108.708); **CAS 170447** (m) - Animas, Hidalgo Co., NM [vi] (31.949, 108.807); **CMNH 68102** (m) - Animas, Hidalgo Co., NM [vi] (31.949, 108.807); **CAS 170469** (f) - Hwy 264 nr jctn with 666, McKinley Co., NM [vi] (35.628, 108.780); **CAS 170486** (f) - Hwy 666, McKinley Co., NM [vi] (35.628, 108.780); **CAS 170485** (m) - Hwy 666, McKinley Co., NM [vi] (35.628, 108.780); **CAS 170488** (m) - Hwy 264 nr jctn with 666, McKinley Co., NM [vi] (35.628, 108.780); **CAS 179466** (m) - hwy 666, McKinley Co., NM [vi] (35.628, 108.780); **UTEP 9011** (m) - Orogrande, 2 mi S on Hwy 54, Otero Co., NM [vi] (32.371, 106.084); **TCWC 33382** (m) - ?, Sierra Co., NM [vi] (33.000, 107.340); **CAS 170514** (f) - West Canyon Road, San Mateo Mountains, Socorro Co., NM [vi] (33.333, 107.500); **CMNH 137909** (f) - fr 478 nr jctn hwy 52, Socorro Co., NM [vi] (34.113, 107.669); **CAS 170515** (m) - San Mateo Mountains, Socorro Co., NM [vi] (33.333, 107.500); **CAS-SU 10884** (m) - San Augustine Plains, Socorro Co., NM [vi] (34.113, 107.669); **CAS 37997** (m) - Lamoiville, Elko Co., NV [lu] (40.728, 115.478); **CAS-SU 21637** (m) - Anahoe Island, Pyramid Lake, Washoe, NV [lu] (40.075, 119.701); **CAS 192647** (f) - Anahoe Island, Pyramid Lake, WashoeNV [lu] (40.075, 119.701); **CAS-SU 21623** (f) - Anahoe Island, Pyramid Lake, Washoe, NV [lu] (40.075, 119.701); **CAS-SU 21624** (f) - Anahoe Island, Pyramid Lake, Washoe,

NV [lu] (40.075, 119.701); **CAS-SU 21626** (f) - Anahoe Island, Pyramid Lake, Washoe, NV [lu] (40.075, 119.701); **CAS-SU 21628** (f) - Anahoe Island, Pyramid Lake, Washoe, NV [lu] (40.075, 119.701); **CAS-SU 21632** (f) - Anahoe Island, Pyramid Lake, Washoe, NV [lu] (40.075, 119.701); **CAS-SU 21638** (f) - Anahoe Island, Pyramid Lake, Washoe, NV [lu] (40.075, 119.701); **CAS-SU 21622** (m) - Anahoe Island, Pyramid Lake, Washoe, NV [lu] (40.075, 119.701); **CAS-SU 21625** (m) - Anahoe Island, Pyramid Lake, Washoe, NV [lu] (40.075, 119.701); **CAS-SU 21627** (m) - Anahoe Island, Pyramid Lake, Washoe, NV [lu] (40.075, 119.701); **CAS-SU 21629** (m) - Anahoe Island, Pyramid Lake, Washoe, NV [lu] (40.075, 119.701); **CAS-SU 21630** (m) - Anahoe Island, Pyramid Lake, Washoe, NV [lu] (40.075, 119.701); **CAS-SU 21631** (m) - Anahoe Island, Pyramid Lake, Washoe, NV [lu] (40.075, 119.701); **CAS-SU 21633** (m) - Anahoe Island, Pyramid Lake, Washoe, NV [lu] (40.075, 119.701); **CAS-SU 21636** (m) - Anahoe Island, Pyramid Lake, Washoe, NV [lu] (40.075, 119.701); **MVZ 12323** (f) - Cherry Creek, White Pine Co., NV [lu] (39.888, 114.854); **MVZ 11373** (m) - Spring Valley, White Pine Co., NV [lu] (39.344, 114.414); **MVZ 11378** (m) - Spring Valley, White Pine Co., NV [lu] (39.344, 114.414); **MVZ 11382** (m) - Spring Valley, White Pine Co., NV [lu] (39.344, 114.414); **MVZ 16254** (m) - Spring Valley, White Pine Co., NV [lu] (39.344, 114.414); **MVZ 24668** (m) - Baker, White Pine Co., NV [lu] (39.013, 114.122); **CMNH 42849** (m) - Knowles, Beaver Co., OK [vi] (36.873, 100.193); **CMNH 91718** (f) - Watonga, Blaine Co., OK [vi] (35.845, 98.413); **CMNH 93835** (f) - Kenton, Cimarron Co., OK [vi] (36.903, 102.963); **CMNH 105545** (f) - 14mi N Woodward Co. line on St hwy 34, Harper Co., OK [vi] (36.937, 99.276); **CMNH 93810** (f) - Hooker, Texas Co., OK [vi] (36.860, 101.213); **CMNH 105550** (m) - Jctn US 64 & st hwy 955, Texas Co., OK [vi] (36.860, 101.213); **USNM 53067** (m) - Agency, Jefferson Co., OR [or] (44.621, 121.160); **CMNH 9846** (f) - 30 mi W Pierre, Stanley Co., SD [vi] (44.354, 100.373); **CMNH 9847** (m) - 30 mi W Pierre, Stanley Co., SD [vi] (44.354, 100.373); **FMNH 14816** (m) - Crane, Crane Co., TX [vi] (31.397, 102.350); **UTEP 14090** (f) - Farm Rd 2185 3.9 mi E jct with Farm Road 2809, Culberson Co., TX [vi] (31.856, 107.000); **UTEP 15429** (m) - Hwy 62/1800 22.8 mi E El Paso Hudspeth Co. line, Hudspeth Co., TX [vi] (31.500, 106.000); **USNM 36342** (m) - Beaver Canyon, Beaver Co., UT [lu] (38.277, 112.640); **UMNH 1304** (m) - Shadscale Flat, 4 mi S. Price, Carbon Co., UT [?](39.599, 110.810); **UMNH 1359** (m) - Shadscale Flat, 4 mi S. Price, Carbon Co., UT [?](39.599, 110.810); **UMNH 1197** (m) - Escalante den, Garfield Co., UT [co] (37.770, 111.601); **UMNH 1198** (m) - Escalante, Garfield Co., UT [co] (37.770, 111.601); **UMNH 1201** (m) - Escalante, Garfield Co., UT [co] (37.770, 111.601); **UMNH 306** (m) - Kings Ranch nr Henry Mountains, Garfield Co., UT [co] (38.061, 110.936); **CAS 40960** (f) - Thompson, Grand Co., UT [co] (38.728, 109.711); **CAS 148648** (m) - Arches Natl Monument, Grand Co., UT [co] (38.616, 109.612); **CAS 38098** (m) - Thompson, Grand Co., UT [co] (38.728, 109.711); **UTEP 5288** (m) - Moab, Grand Co., UT [co] (38.573, 109.549); **USNM 36344** (f) - Buckhorn Hills, Iron Co., UT [lu] (38.340, 113.839); **UMNH 2237** (m) - Fish Springs, Juab Co., UT [lu] (39.839, 113.398); **CAS 170153** (f) - Emigration Canyon, entrance to Hogle Zoo, Salt Lake Co., UT [lu] (40.751, 111.810); **CAS 170478** (f) - Emigration Canyon, Hogle Park Zoo, Salt Lake Co., UT [lu] (40.751, 111.810); **CAS 170480** (f) - Emigration Canyon, Hogle Park Zoo, Salt Lake Co., UT [lu] (40.751, 111.810); **CAS 170481** (f) - Entrance to Hogle Park Zoo, Emigration Canyon, Salt Lake Co., UT [lu] (40.751, 111.810); **CAS 170512** (f) - Entrance to Hogle Park Zoo, Emigration Canyon, Salt Lake Co., UT [lu] (40.751, 111.810); **CAS 170479** (m) - Emigration Canyon, Hogle Park Zoo, Salt Lake Co., UT [lu] (40.751, 111.810); **CAS 30922** (m) - Fort Douglas, Salt Lake Co., UT [lu] (40.764, 111.831); **CAS 170411** (f) - Moab, San Juan Co., UT [co] (38.573, 109.549); **CAS 170416** (f) - Moab, San Juan Co., UT [co] (38.573, 109.549); **CAS 170434** (f) - Moab, San Juan Co., UT [co] (38.573, 109.549); **CAS 179410** (f) - Moab, San Juan Co., UT [co] (38.573, 109.549); **UTEP 15091** (f) - Monticello, 10 mi N on Hwy 160, San Juan Co., UT [vi] (31.500, 104.500); **CAS 170409** (m) - Moab, San Juan Co., UT [co] (38.573, 109.549); **CAS 170417** (m) - Moab, San Juan Co., UT [co] (38.573, 109.549); **CAS 170426** (m) - Moab, San Juan Co., UT [co] (38.573, 109.549); **CAS 170435** (m) - Moab, San Juan Co., UT [co] (38.573, 109.549); **CMNH 11580** (m) - Monument Valley, 35mi SW Bluff, San Juan Co., UT [co] (37.140, 110.207); **MVZ 17891** (m) - Blanding, Alkali Mesa, San Juan Co., UT [co] (37.624, 109.478); **UMNH 1134** (m) - Between Moab and Monticello, San Juan Co., UT [co] (37.871, 109.342); **UMNH 2199** (f) - Coalville, Summit Co., UT [lu] (40.918, 111.399); **UMNH 2202** (f) - Coalville, Summit Co., UT [lu] (40.918, 111.399); **UMNH 2217** (f) - Coalville, Summit Co., UT [lu] (40.918, 111.399); **UMNH 2106** (m) - Coalville, Summit Co., UT [lu] (40.918, 111.399); **UMNH 2205** (m) - Coalville, Summit Co., UT [lu] (40.918, 111.399); **UMKC 17784** (f) - Delta, Tooele Co., UT [lu] (39.352, 112.576); **UMNH 1976** (f) - Lowe, Tooele Co., UT [lu] (40.786, 112.940); **UMNH 1993** (f) - Lowe, Tooele Co., UT [lu] (40.786, 112.940); **UMNH 1994** (f) - Lowe, Tooele Co., UT [lu] (40.786, 112.940); **UMNH 1972** (m) - Lowe, Tooele Co., UT [lu] (40.786, 112.940); **UMNH 1975** (m) - Lowe, Tooele Co., UT [lu] (40.786, 112.940); **UMNH 1977** (m) - Lowe, Tooele Co., UT [lu] (40.786, 112.940); **UMNH 2022** (m) - Grantsville, Tooele Co., UT [lu] (40.600, 112.464); **UTEP 16592** (m) - Skull Valley, Tooele Co., UT [lu] (40.746, 112.691); **UMNH 2833** (m) - Vernal, Uintah Co., UT [co] (40.456, 109.528); **CAS 170462** (m) - Jensen, Uintah Co., UT [co] (38.724, 112.098); **UMNH 2852** (m) - Vernal, Uintah Co., UT [co] (40.456, 109.528); **CAS 170427** (f) - Provo, Utah Co., UT [lu] (40.234, 111.658); **CAS 170429** (m) - Provo, Utah Co., UT [lu] (40.234, 111.658); **USNM 310863** (m) - St. George, Washington Co., UT [lu] (37.104, 113.583); **CMNH 39822** (m) - Othello, Adams Co., WA [or] (46.826, 119.174); **CMNH 32722** (f) - Ephrata, Grant Co., WA [or] (47.318, 119.553); **CAS 170431** (f) - Ellensburg, Kittitas Co., WA [or] (46.997, 120.547); **CAS 170436** (f) - Ellensberg, Kittitas Co., WA [or] (46.997, 120.547); **CAS 170437** (f) - Ellensburg, Kittitas Co., WA [or] (46.997, 120.547); **CAS 170439** (f) - Ellensburg, Kittitas Co., WA [or] (46.997, 120.547); **CMNH 32731** (f) -

Klickitat, Klickitat Co., WA [or] (45.817, 121.151); Z 04954 (f) - Pateros, Okanogan Co., WA [or] (48.051, 119.902); Z 4508 (m) - Pateros, Okanogan Co., WA [or] (48.051, 119.902); Z 4549 (m) - Pateros, Okanogan Co., WA [or] (48.051, 119.902); Z 4553 (m) - Pateros, Okanogan Co., WA [or] (48.051, 119.902); Z 4554 (m) - Pateros, Okanogan Co., WA [or] (48.051, 119.902); Z 4556 (m) - Pateros, Okanogan Co., WA [or] (48.051, 119.902); Z 8443 (m) - Pateros, Okanogan Co., WA [or] (48.051, 119.902); CMNH 91517 (f) - Wawawai, Whitman Co., WA [or] (46.637, 117.378); CMNH 91572 (f) - Wawawai, Whitman Co., WA [or] (46.637, 117.378); CMNH 91503 (m) - Wawawai, Whitman Co., WA [or] (46.637, 117.378); UMKC 87593 (f) - Hyattville, Big Horn Co., WY [vi] (44.246, 107.602); UMKC 27832 (m) - Spotted Horse, Campbell Co., WY [vi] (44.708, 105.834); UMKC 27835 (m) - Gillette, Campbell Co., WY [vi] (44.291, 105.502); UMKC 27836 (m) - Gillette, Campbell Co., WY [vi] (44.291, 105.502); CMNH 93877 (f) - 21.5mi N Sinclair on rd to Seminoe Dam, Carbon Co., WY [vi] (41.775, 107.113); CAS 170442 (m) - Moneta, Fremont Co., WY [vi] (43.162, 107.724); CAS 170470 (m) - Rivertons, Fremont Co., WY [vi] (43.025, 108.379); UMKC 79710 (f) - Goshen, Goshen Co., WY [vi] (42.083, 104.350); UMKC 98072 (f) - Alcova, Natrona Co., WY [vi] (42.552, 106.716); UMKC 63778 (m) - Moore Springs, Niobrara Co., WY [vi] (43.340, 104.500); UMKC 79711 (m) - Alcova, Niobrara Co., WY [vi] (42.552, 106.716); CAS 170477 (f) - Cody, Park Co., WY [vi] (44.526, 109.056); CAS-SU 11613 (m) - 20mi E of Cady Hill, N of US hwy 14, Park Co., WY [vi] (44.526, 109.056); CMNH 105639 (f) - Glendo Dam, Platte Co., WY [vi] (42.480, 104.948); CMNH 93898 (f) - Glendo Lake spillway, Platte Co., WY [vi] (42.467, 104.967); CMNH 105591 (m) - Hartville, Platte Co., WY [vi] (42.328, 104.725); CMNH 105592 (m) - Hartville, Platte Co., WY [vi] (42.328, 104.725); CMNH 105640 (m) - Glendo Dam, Platte Co., WY [vi] (42.480, 104.948); FMNH 37208 (m) - Guernsey, Platte Co., WY [vi] (42.270, 104.741); CAS 170459 (f) - Flaming Gorge Reservoir, Sweetwater Co., WY [co] (40.914, 109.421); CAS 170461 (f) - Firehole Road, Sweetwater Co., WY [co] (40.914, 109.421); CAS 170464 (f) - Flaming Gorge Reservoir, Sweetwater Co., WY [co] (40.914, 109.421); CAS 170465 (f) - Firehole Road, Sweetwater Co., WY [co] (40.914, 109.421); CMNH 21978 (f) - 15mi SE Bitter Creek, Haystack Mountain, Sweetwater Co., WY [vi] (41.518, 109.450); CAS 170460 (m) - Flaming Gorge Reservoir, Sweetwater Co., WY [co] (40.914, 109.421); CAS 170463 (m) - Flaming Gorge Reservoir, Sweetwater Co., WY [co] (40.914, 109.421); CAS 170500 (m) - Flaming Gorge, Sweetwater Co., WY [co] (40.914, 109.421); CMNH 21979 (m) - 15mi SE Bitter Creek, Haystack Mountain, Sweetwater Co., WY [vi] (41.518, 109.450); CMNH 21980 (m) - 15mi SE Bitter Creek, Haystack Mountain, Sweetwater Co., WY [vi] (41.518, 109.450); UMKC 23596 (m) - Flaming Gorge Reservoir, Sweetwater Co., WY [co] (40.914, 109.421).

Appendix V. Selection of geographically variable characters for transects (Chapter 3, Section 3.2.5)

Table V.1. *F*-values of 2-way ANOVA (meristic characters) showing morphological characters vary between 1) geographical locality (group), 2) sex, and/or 3) interaction between locality and sex. Significant characters are marked as follows: $P<0.01$**, $P<0.05$*.

Character	2-way ANOVA *F*-values					
	Males			Females		
	CA	SER	Idaho	CA	SER	Idaho
Lepidosis						
No. dscr at 10% of body length		9.235*			5.630*	
No. dscr at 20% of body length		14.057*			5.351*	
No. dscr at 30% of body length		18.031*			5.115*	
No. dscr at 40% of body length		11.685*			9.525*	3.227**
No. dscr at 50% of body length		12.621*			11.984*	5.004*
No. dscr at 60% of body length		13.743*			12.789*	2.737**
No. dscr at 70% of body length	2.905**	10.809*			10.568*	
No. dscr at 80% of body length		8.134*			14.778*	
No. dscr at 90% of body length		15.603*		3.885*	7.979*	4.769*
No. dscr at 100% of body length	2.676**	17.983*			9.909*	
No. dscr at 10% of tail length		11.827*		2.904**		
No. dscr at 25% of tail length		11.871*		2.513**	6.260*	3.424**
No. dscr at 75% of tail length		5.491*	2.657*		6.879*	
No. ventral scales	3.210*	8.800*	8.920*		9.368*	10.906*
No. subcaudal scales			3.694**			
No. supralabials			5.095*	2.439**		6.920*
No. infralabials				2.994**		3.404**
No. intersupraoculars		3.394**	6.835*		4.707*	3.587**
No. interoculabials	3.771*	2.763**	3.697**		3.156**	
No. prefoveals	2.969**	3.412**	5.825*	3.284*	6.517*	5.7373*
General pattern						
No. dorsal blotches	6.353*		24.571*	2.684**	3.587**	20.660*
No. tail bands		3.240**	23.929*			7.700*
% dorsal blotches fused with lateral	10.035*	4.388*	3.009**	7.724*		8.899**
Amount of ventral mottling			3.534**	2.856**		8.116*
Mean length of blotch margin at 25%			17.558*	4.084*	2.770**	28.197*
Mean length of blotch margin at 50%	4.118*		20.438*	4.246*		17.273*
Mean length of blotch margin at 75%	2.431**		17.962*	2.962**	2.594**	17.620*
Mean blotch midline length at 25%			16.558*	4.868*		42.989*
Mean blotch midline length at 50%			24.097*			18.398*
Mean blotch midline length at 75%	4.234*			2.962**	4.046*	31.910*
Blotch width at 25%		3.457**		2.860**		
Blotch width at 50%		7.140*			3.299**	
Blotch width at 75%	13.591*	5.236*	3.012**	8.668*		6.893*

Table V.2. *T*-values of 2-way ANCOVA (mensural characters) showing morphological characters vary between 1) geographical locality (group), 2) sex, and/or 3) interaction between locality and sex. Significant characters are marked as follows: $P<0.01$**, $P<0.05$*.

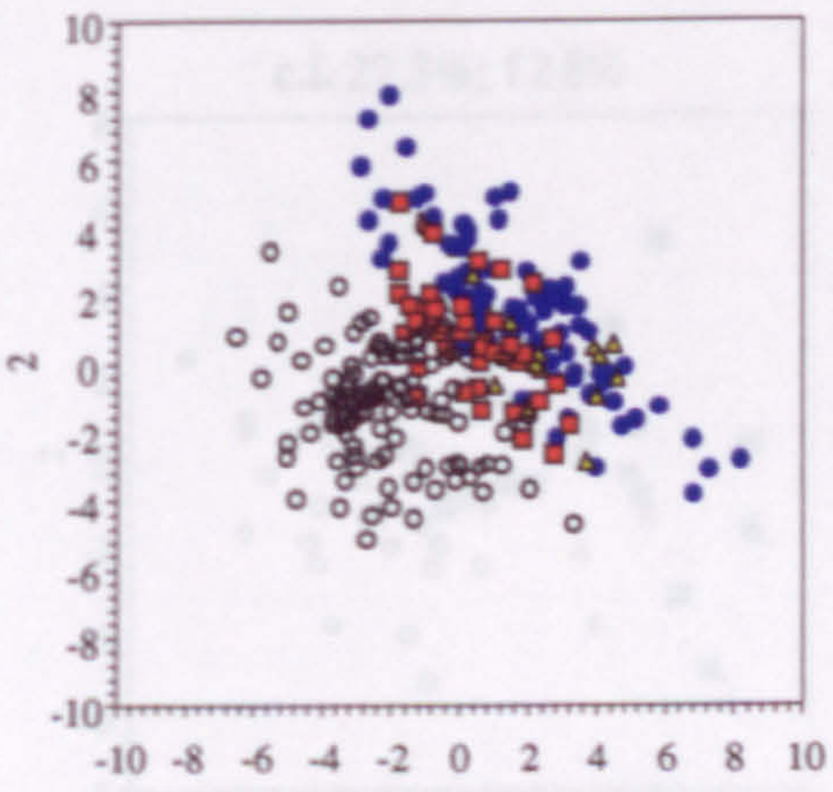
Character	2-way ANCOVA *T*-values					
	Males			Females		
	CA	SER	Idaho	CA	SER	Idaho
Body dimensions						
Tail length			15.873*		13.849*	
Head length			12.701*			8.788*
Head width (widest point)		12.765*				9.515*
Head width at eye level	12.006*	12.396*	10.275*			
Snout length				14.266*		7.927*
Distance: eye to nostril						
Distance: eye to pit				7.302*		
Distance: nostril to pit		12.720*	6.034*			
General pattern				7.816*		5.437*
Blotch length at 25% (mm)	10.165*					5.878*
Blotch length at 50% (mm)	10.271*					8.953*
Blotch length at 75% (mm)	10.003*					
Other dimensions					3.361*	
Proximal rattle length				17.458*		13.370*
Proximal rattle depth		22.256*				3.932*
Proximal rattle width	8.048*	11.057*		12.101*		
Eye diameter		9.922*		9.783*		
Pit diameter			7.625*			11.243*
Mental scale width		12.839*				8.598*
Rostral scale length		12.743*	9.208*			
Rostral scale width		11.428*				8.356*
Prenasal scale length	13.954*					7.546*
Prenasal scale width		8.678*				6.833*
Supraocular scale length	12.220*		10.084*			8.413*
Supraocular scale width	9.756*	15.166*	9.859*			

Appendix VI. Selection of OTUs (Chapter 3)

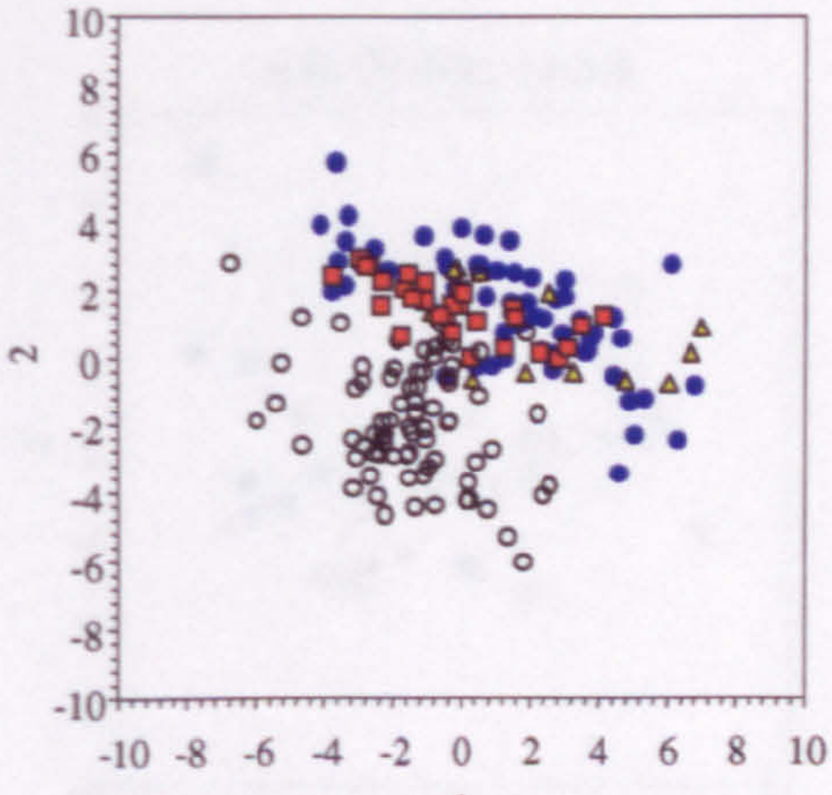
Scatter plots of the second against first principal component scores representing all meristic characters (refer to Chapter 3, for details), used to define operational taxonomic units. Plot (i) depicts males and (ii) females. The percentages above each plot represent variation expressed by the first and second component scores respectively.

Two groups are revealed (a.i and a.ii):1. Pacific coastal forms (*helleri, oreganus, caliginis*) and *cerberus* from the Arizona highlands, 2. individuals from south and east of the Rocky Mts. (*viridis*), the Colorado Plateau (*nuntius, concolor*) and the Great Basin (*lutosus*). When the Pacific Coastal forms are excluded, the Great Basin individuals depart from the eastern and Colorado Plateau forms (figs. b.i and b.ii).

a.i. 23.8%; 13.5%



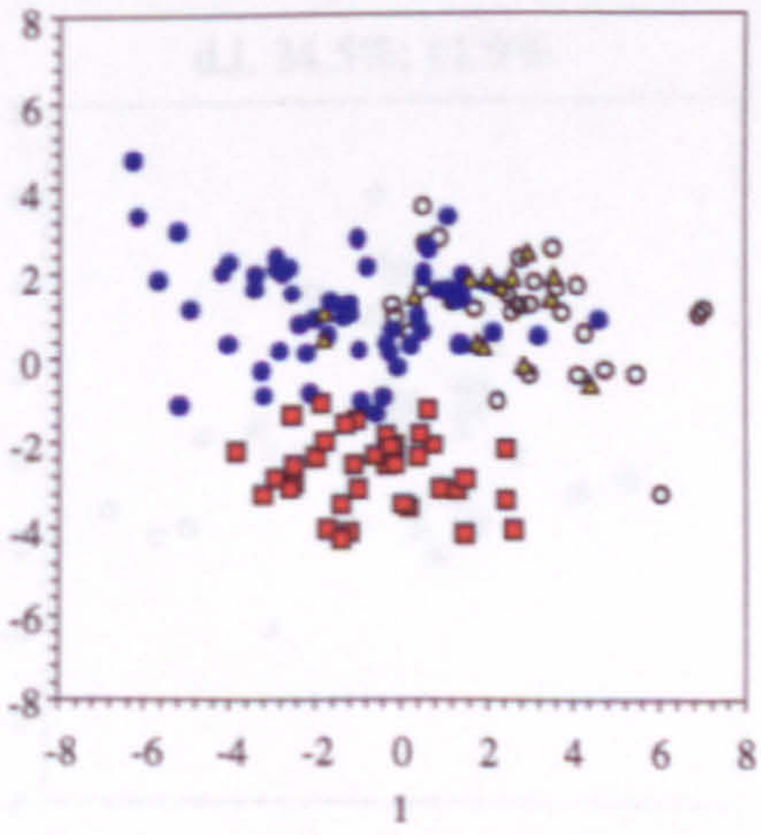
a.ii. 24.9%; 14.7%



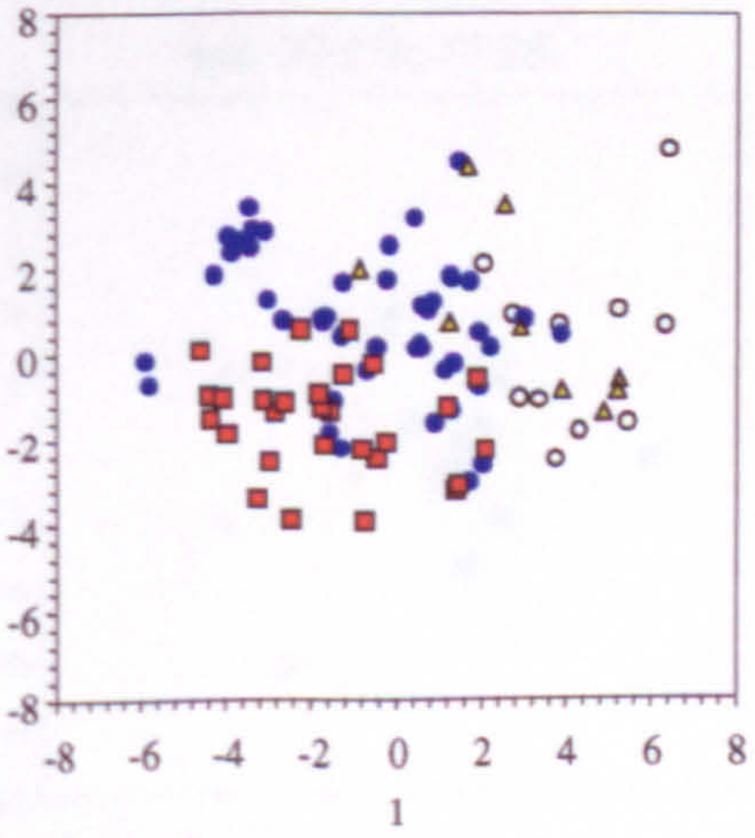
All individuals

- East and south of Rocky Mountains - *viridis* and Colorado Plateau - *nuntius*
- Colorado Plateau - *concolor*
- Arizona highlands - *cerberus*, Pacific Coast - *helleri, oreganus*; Los Coronados - *caliginis*
- Great Basin - *lutosus*

b.i. 23.6%; 17.6%



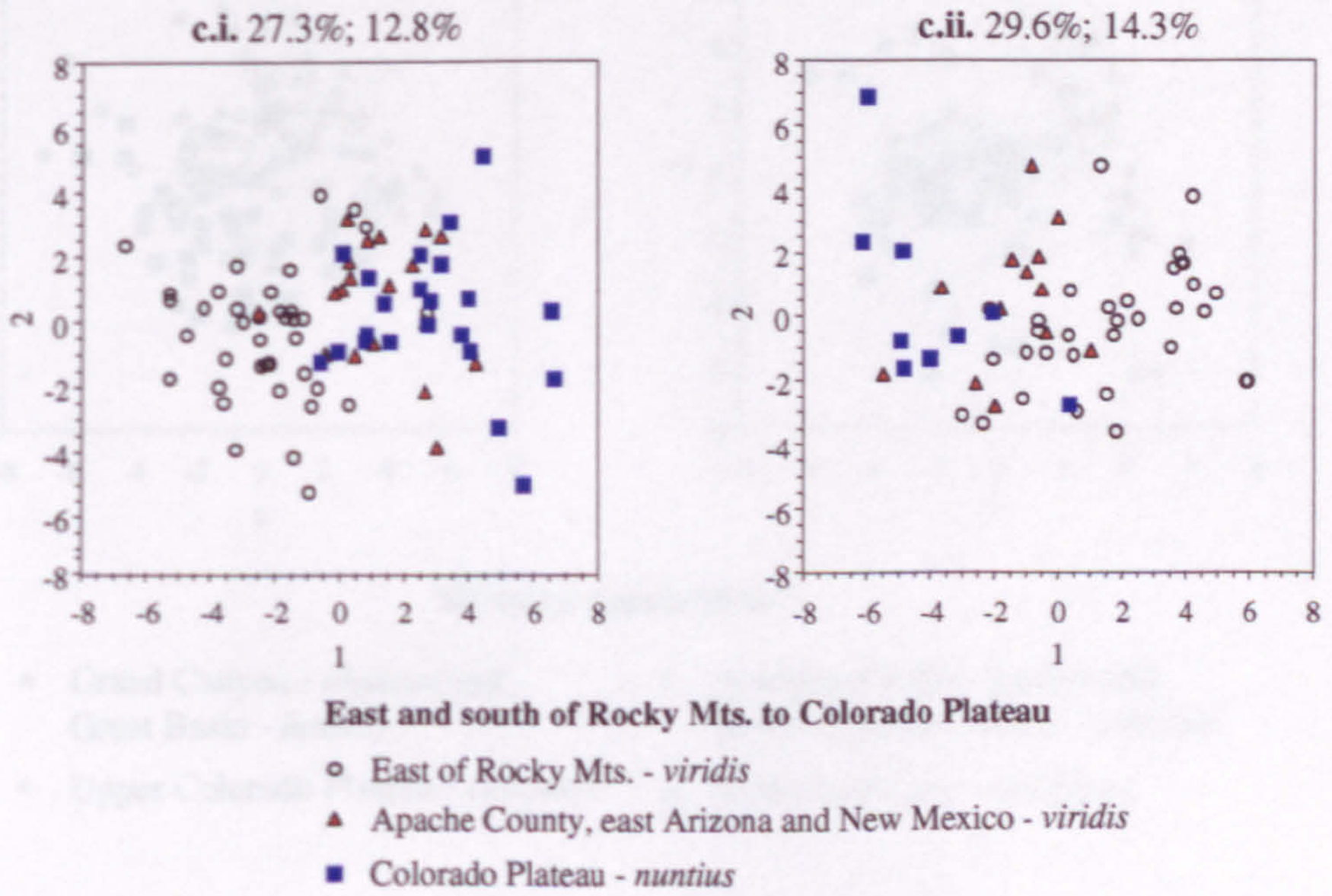
b.ii. 23.5%; 18.4%

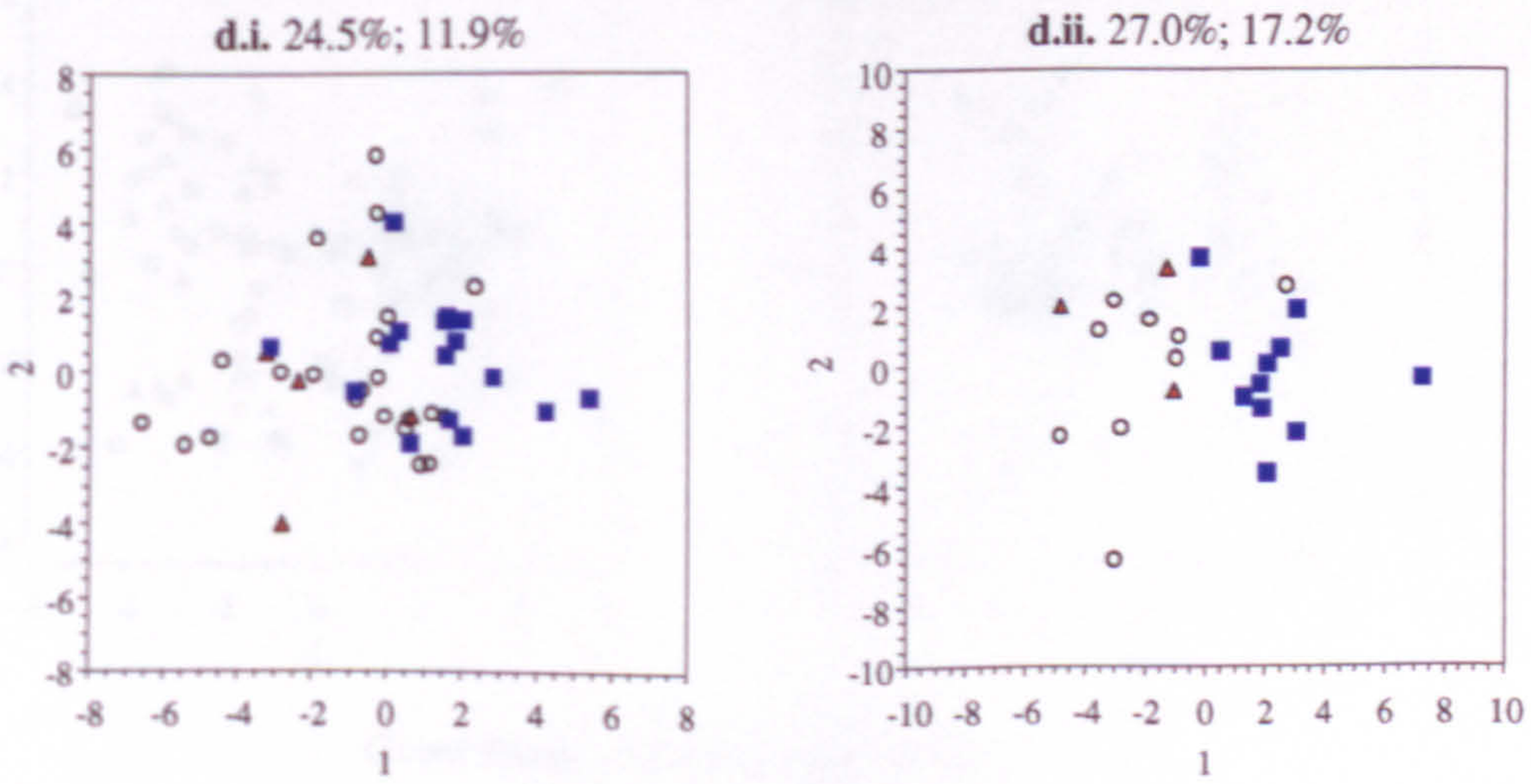


Pacific Coast and Arizona Highlands (*cerberus*) excluded

- East and south of Rocky Mountains - *viridis*
- Colorado Plateau - *concolor*
- Colorado Plateau - *nuntius*
- Great Basin - *lutosus*

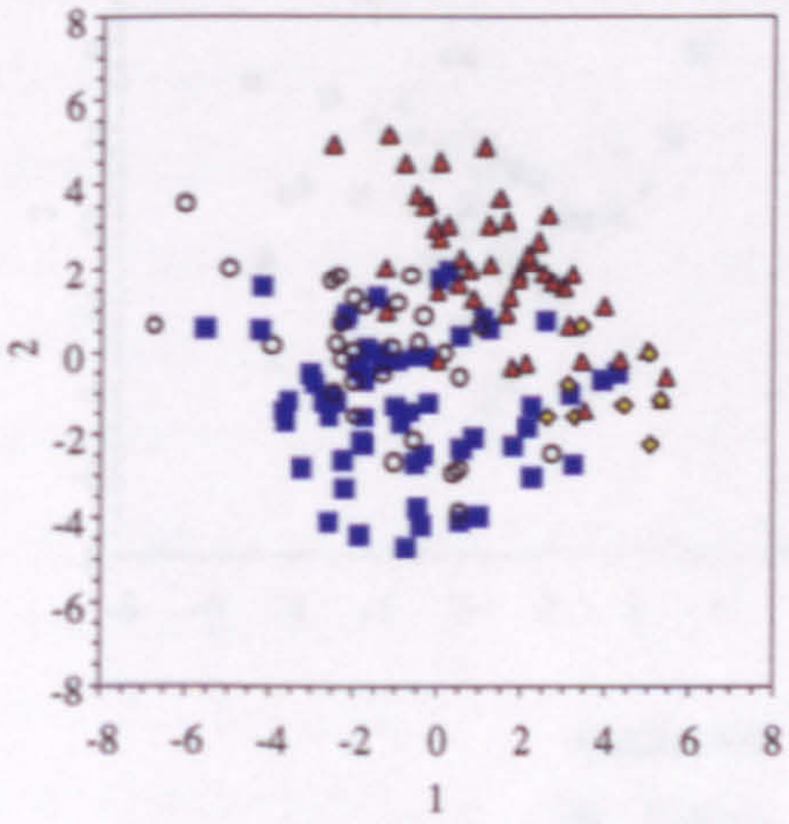
Individuals from Montana, south through Texas to the New Mexico border (currently *C. v. viridis*) are outwardly distinct from the Colorado Plateau forms (currently *C. v. nuntius*), but show a degree of gradual change in first principal component score moving towards the Apache County (Arizona) and Colorado Plateau individuals (figs. c.i and c.ii). The relationship of the New Mexico, Arizona and Texas *viridis-nuntius* individuals is not clear. In the males there is weak separation of the Colorado Plateau *nuntius* from *C. v. viridis*, with considerable overlap, while in the females this separation is more distinct. In both instances the Apache County *nuntius* are tending to group with the Colorado Plateau forms (figs. d.i and d.ii).



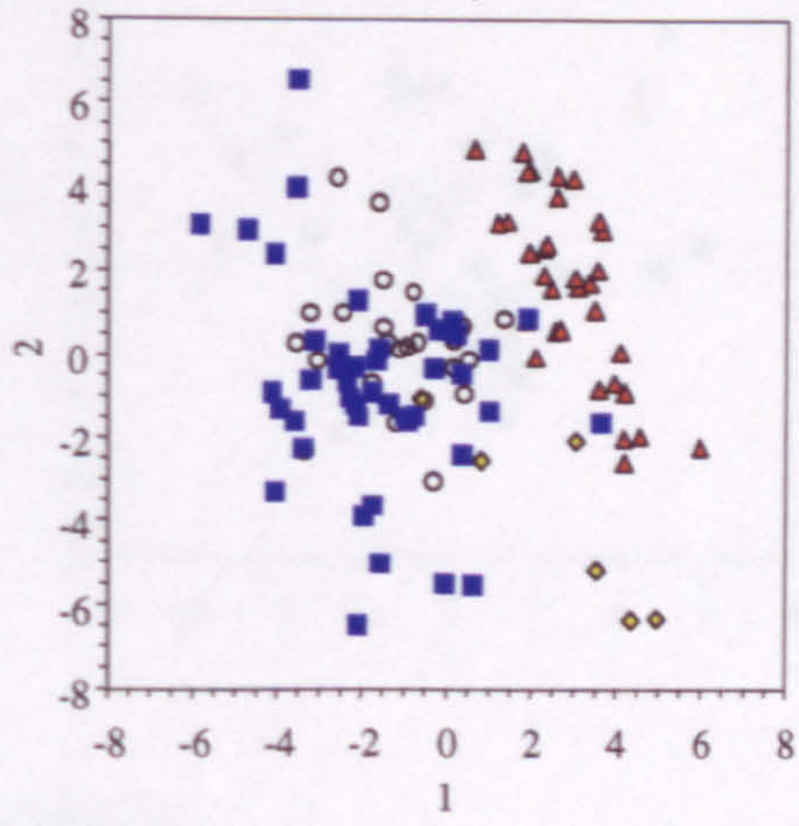


The western populations form three regional groups, the Pacific Coast, Great Basin, and Colorado -Green River basins of the Colorado Plateau (e.i and e.ii). No further structuring occurs within the GB group, except the Anahoe Island population differs slightly from the mainland. Males from Adams and Nez Perce Counties, Idaho (a *C. v. oreganus* and *C. v. lutosus* contact zone) tend to group with GB *lutosus* and PC *oreganus* respectively (figure f.i). Among females, a clear PC-GB split is clear, although the females are not represented by individuals from the contact zone (f.ii).

e.i. 19.1%; 13.7%



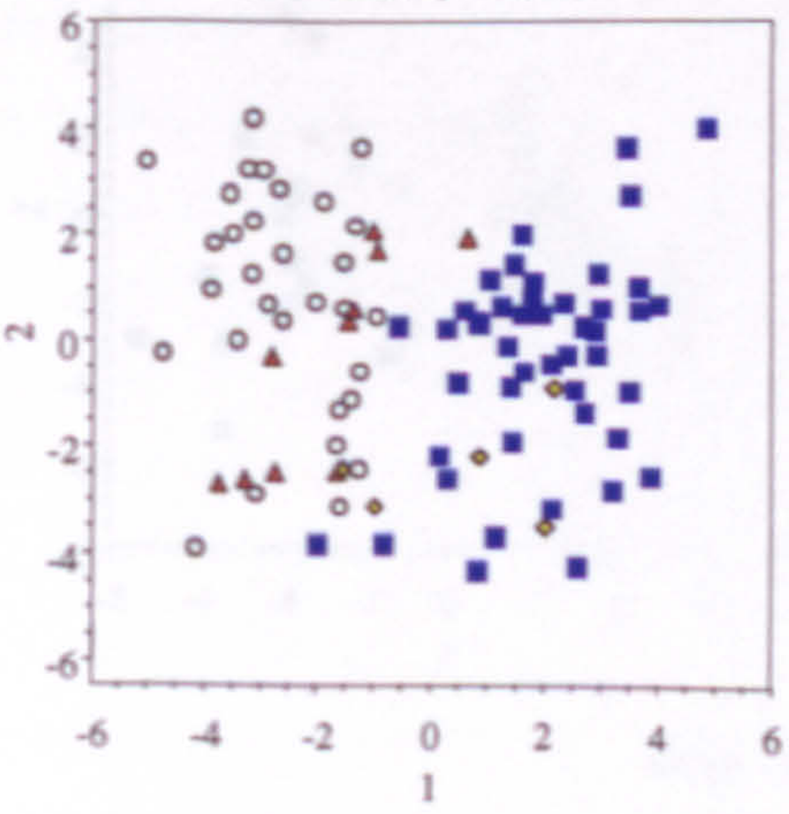
e.ii. 21.3%; 19.5%



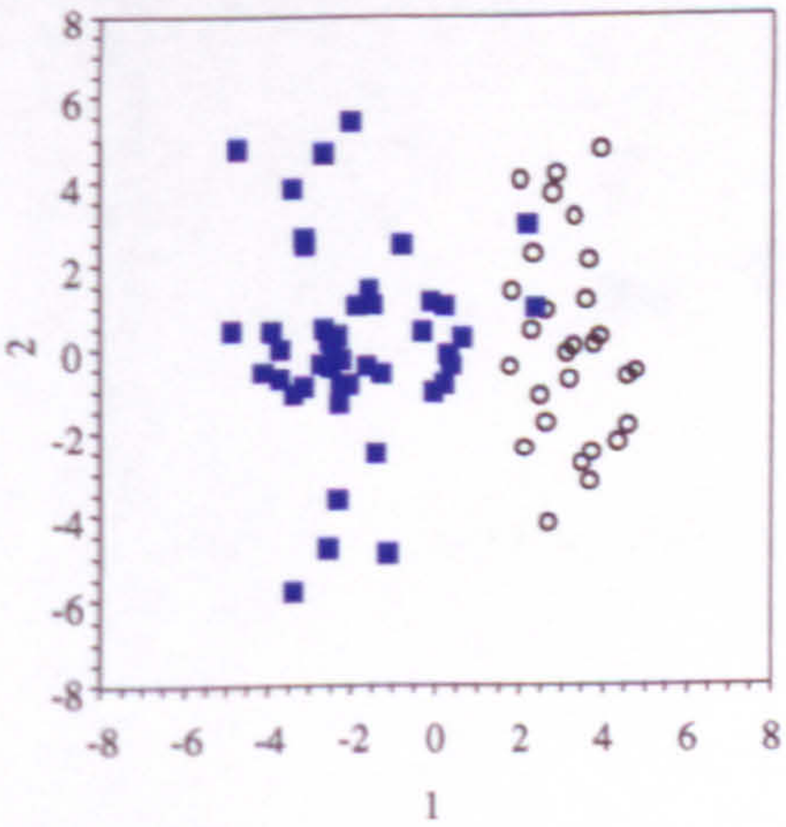
Western populations

- ▲ Grand Canyon - *abyssus* and Great Basin - *lutosus*
- ◆ Upper Colorado Plateau - *concolor*
- ○ Southern Pacific - *helleri* and South Coronado Island - *caliginis*
- ■ Northern Pacific - *oreganus*

f.i. 18.2%; 13.8%



f.ii. 22.4%; 14.5%

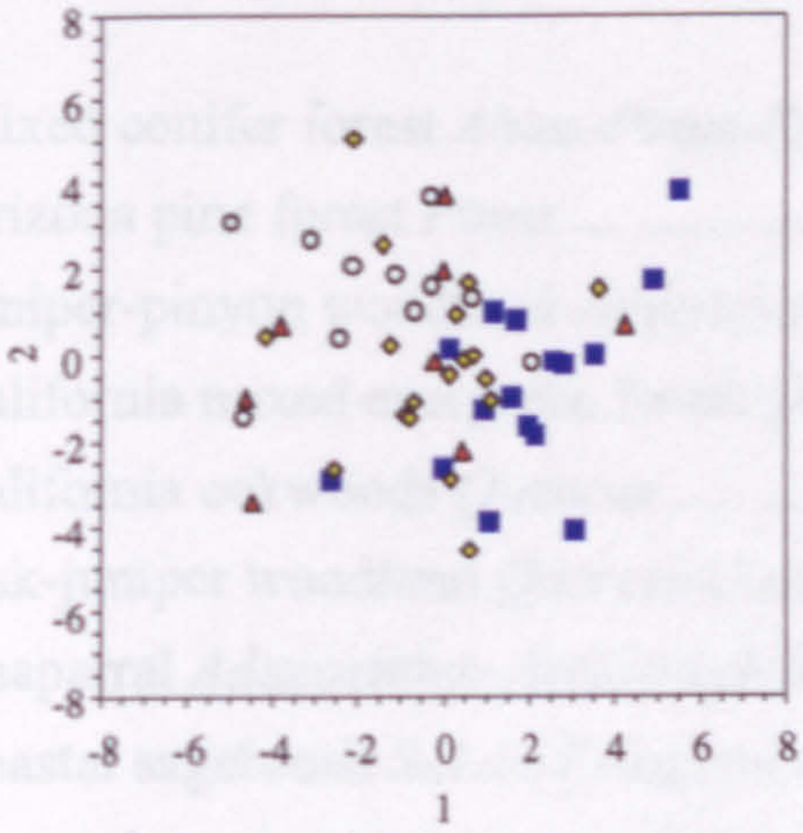


Great Basin and Pacific northwest

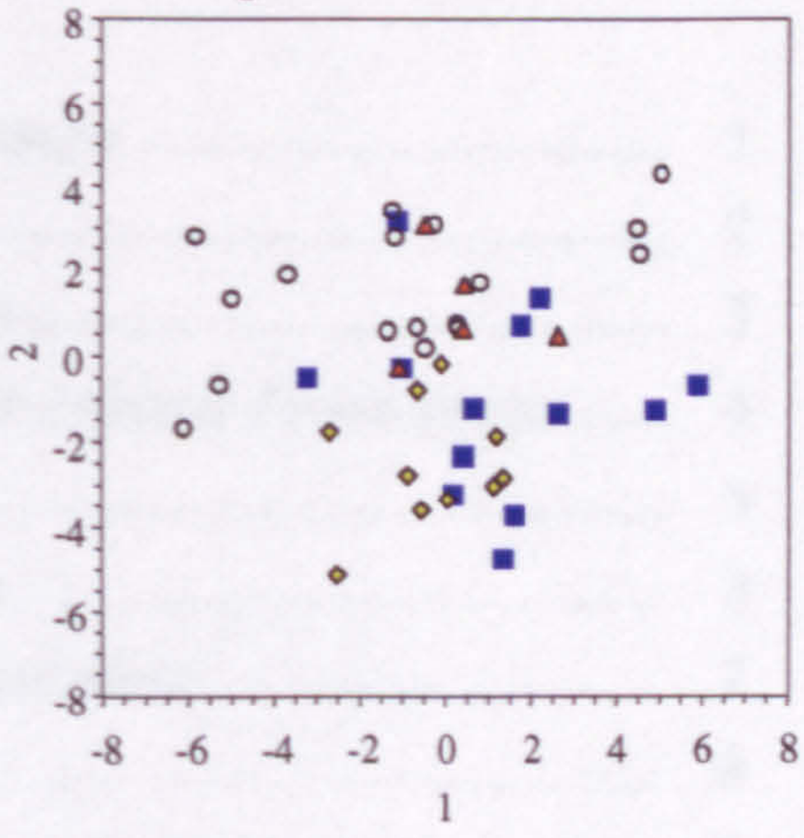
- ○ Utah, Idaho and Nevada - *lutosus*
- ▲ Idaho (Adams County) - intermediate
- ◆ Idaho - intermediate
- ■ California, Washington and Canada - *oreganus*

Both *oreganus* and *helleri* morphological types have been identified from Santa Barbara, Monterey and San Luis Obispo Counties, which overlap with other Californian central states in the PCA plot (g.i and g.ii). Discrete groups are confirmed for *C. v. cerberus*, *C. v. abyssus*, and *C. v. viridis-C.v.nuntius* respectively (h.i and h.ii).

g.i. 20.3%; 13.8%



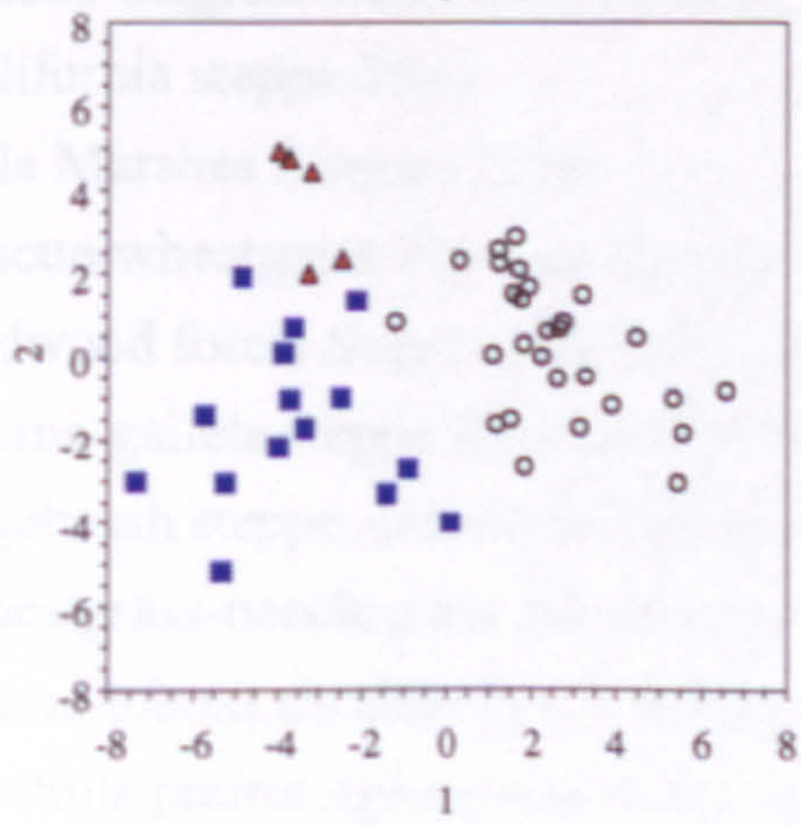
g.ii. 24.4%; 18.8%



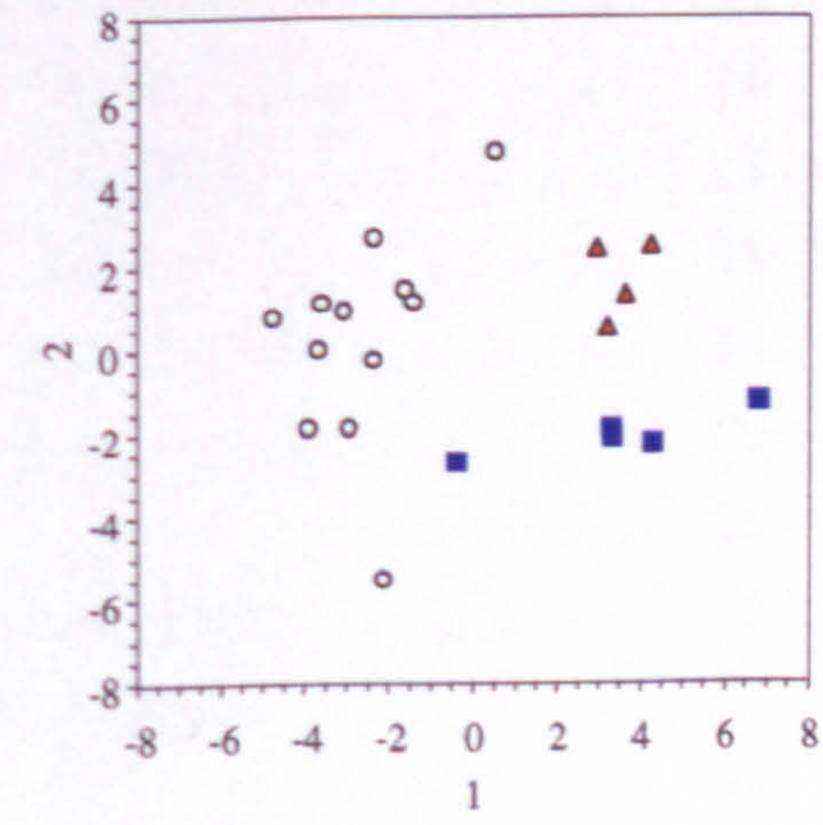
California

- *helleri* - southern California
- *oreganus* - northern California
- *?* - central valley
- *?* - Monterey and San Luis Obispo Counties

h.i. 36.6%; 15.8%



h.ii. 36.4%; 16.5%



Arizona

- *abyssus* - Grand Canyon
- *cerberus*
- *nuntius* - Colorado Plateau
  viridis - Apache County

Appendix VII. Vegetation categories

Vegetation categories used in testing causal hypotheses of morphological pattern in *Crotalus viridis*. Extracted from the National Atlas of the United States of America, United States Department of the Interior Geological Survey, 1970.

Mixed conifer forest *Abies-Pinus-Pseudotsuga* 1
Arizona pine forest *Pinus*..... 2
Juniper-pinyon woodland *Juniperus-Pinus* 3
California mixed evergreen forest *Quercus-Arbutus-Pseudotsuga*..... 4
California oakwoods *Quercus* 5
Oak-juniper woodland *Quercus-Juniperus* 6
Chaparral *Adenostoma-Arctostaphylos-Caenothus* 7
Coastal sagebrush *Salvia-Eriogonum* 8
Mountain mahogany-oak scrub *Cercocarpus-Quercus* 9
Great Basin sagebrush *Artemisia* 10
Saltbrush-greasewood *Atriplex-Sarcobatus.* 11
Creosote bush *Larrea* 12
Creosote bush-bur sage *Larrea-Franseria* 13
Desert - vegetation largely absent..... 14
Fescue-oatgrass *Festuca-Danthonia* 15
California steppe *Stipa* 16
Tule Marshes *Scirpus-Typha* 17
Fescue-wheatgrass *Festuca-Agropyron* 18
Redwood forest *Sequoia-Pseudotsuga*..... 19
Grama-galleta steppe *Bouteloua-Hilaria* 20
Sagebrush steppe *Artemisia-Agropryon* 21
Wheatgrass-needlegrass shrubsteppe *Agropyron-Stipa-Artemisia*..... 22
Grama-tobosa shrubsteppe *Bouteloua-Hilaria-Larrea*..... 23
Foothills prairie *Agropyron-Festuca-Stipa* 24
Grama-needlegrass-wheatgrass *Bouteloua-Stipa-Agropyron*..... 25
Grama-buffalo grass *Bouteloua-Buchloe* 26
Wheatgrass-needlegrass *Agropyron-Stipa* 27
Nebraska sandhills prairie *Andropogon-Calamovilfa* 28

Appendix VIII. Results of pairwise Mantel tests of association between morphology and cause Chapter 4

** = $P<0.01$; * = $P<0.05$; grey shaded = non-significant

All males n=236

Character	Patristic distance	Vegetation	Monthly temperature	Monthly rainfall
genmorph	0.2602**	0.1542**	0.1321**	0.1430**
headshapee	0.0649**	0.0237	0.1249**	-0.0124
headsize	0.0662**	0.0103	0.0856**	0.0020
genpat	0.3393**	0.1513**	0.1387**	0.1908**
genblotb	0.1747**	0.1010**	0.1414**	0.1197**
genblotc	0.2232**	0.1329**	0.1422**	0.1218**
genblotd	0.1750**	0.0900**	0.0199	0.1103*
genblotw	0.2049**	-0.0879**	-0.0017	0.0629*
dblot	0.2624**	0.1133**	0.1245**	0.2470**
tblot	0.3839**	0.1126**	0.1451**	0.2295**
ventmot	0.4952**	0.1284**	0.9057**	0.1486**
genscut	0.1845**	0.1019**	0.0523	0.0746*
genheadsc	0.2106**	0.0866**	0.0311	0.5584*
ventrals	0.0353**	0.0549**	0.0246	0.0675*
dorsrows	0.0886**	0.0580**	-0.0011	0.0347
tailrows	0.1537**	0.0711**	-0.0147	0.0355

All females n=175

Character	Patristic distance	Vegetation	Monthly temperature	Monthly rainfall
genmorph	0.1593**	0.1470**	0.1278**	0.2068**
headshape	0.0381**	0.0573**	0.1529**	0.171**
headsize	0.0526**	0.0548**	0.1648**	0.1510**
genpat	0.1801**	0.1254**	0.1284**	0.2162**
genblotb	0.0637**	0.0588**	0.0828*	0.1358**
genblotc	0.0837**	0.0804**	0.0690	0.9449*
genblotd	0.1588**	0.1393**	0.1154**	0.2234**
genblotw	0.0631**	0.0128	-0.0443	0.0172
dblot	0.1451**	0.1180**	0.1915**	0.2664**
tblot	0.1656**	0.1091**	0.1079**	0.1590**
ventmot	0.2433**	0.0767**	0.0475**	0.1010**
genscut	0.1168**	0.0966**	-0.0158	0.0537
genheadsc	0.0944**	0.0300	0.0032	0.0059
ventrals	0.0532**	0.0503**	0.0473	0.1151**
dorsrows	0.0693**	0.0937**	0.0020	0.0790*
tailrows	-0.0791**	0.0108**	-0.0824*	-0.0575

Eastern males n=95

Character	Patristic distance	Geographic position	Vegetation	Monthly temperature	Monthly rainfall
genmorph	0.1756**	0.1935**	0.1726**	0.1638**	0.2101**
headshape	-0.0235	-0.0347	0.0196	0.1017	-0.0724
headsize	-0.0114	-0.0186	0.0109	0.1266	-0.0491
genpat	0.0147	0.0577	0.0382	0.0140	0.0177
genblotb	0.0654	0.0055	0.0155	0.0547	0.0012
genblotc	0.0139	0.0118	0.0595	0.0442	0.0814
genblotd	0.0022	0.0695	0.0232	0.0040	-0.0123
genblotw	0.1160**	0.1142*	0.0560	0.0977*	0.0550
dblot	0.0059	0.0199	0.0247	0.0100	0.0089
tblot	-0.0110	0.0682	0.0014	-0.0261	0.0260
ventmot	0.0127	0.1166**	0.0624	-0.0267	0.0414
genscut	0.1953**	0.2709**	0.1870**	0.1024*	0.3223**
genheadsc	0.0235	0.1306*	0.1167**	0.0828	0.1312*
ventrals	0.0956**	0.0390	0.0427	-0.0284	0.0822
dorsrows	0.1959**	0.2219**	0.1369**	0.1016*	0.2593*
tailrows	0.1586**	0.2429**	0.1477**	0.0720	0.2943**

Eastern females n=63

Character	Patristic distance	Geographic position	Vegetation	Monthly temperature	Monthly rainfall
genmorph	0.0484	0.2794**	0.1639**	0.0389	0.2924**
headshape	0.0498	0.9014**	0.0027	0.1151	0.1872**
headsize	0.0275	0.2152**	0.0281	0.1313	0.1897**
genpat	0.0570	0.0850	0.0168	0.0264	0.0813
genblotb	0.0604	0.0514	0.0082	0.0168	0.0430
genblotc	0.0647	-0.0359	-0.0047	0.0452	0.0393
genblotd	0.0044	0.0178	-0.0261	0.0514	0.0423
genblotw	0.0923**	-0.0095	0.0510	0.0213	0.0138
dblot	-0.0095	0.1776**	0.0413	0.1183**	0.2104**
tblot	-0.0029	0.0509	-0.0329	-0.0730	0.0491
ventmot	0.6494**	0.1149**	0.0310	-0.0367	0.0326
genscut	0.0283	0.2046**	0.2301**	-0.0542	0.2449**
genheadsc	0.0147	0.0427	0.0799**	-0.0240	0.0659
ventrals	-0.0320	0.1217**	0.1318**	-0.0462	0.1547**
dorsrows	0.0098	0.2452**	0.2221**	-0.0286	0.2886**
tailrows	0.0775**	0.0370	0.0795**	-0.0788	0.0141

Western males n=141

Character	Patristic distance	Geographic position	Vegetation	Monthly temperature	Monthly rainfall
genmorph	0.2066**	0.1046**	0.1762**	0.1757**	0.0993*
headshapee	0.0331	-0.0209	0.0277	0.1221**	0.0033
headsize	-0.0029	-0.0319	0.0147	0.0531	-0.0231
genpat	0.2584**	0.1348**	0.1742**	0.1313**	0.1006**
genblot1	0.1861**	0.0501	0.1059**	0.0830**	0.0259
genblot2	0.1639**	0.0784*	0.1191**	0.1194**	0.0499
genblot3	0.2547**	0.2578**	0.1741**	0.1263**	0.1789**
genblotw	0.0975**	0.1264**	0.0622**	0.0649*	0.0444*
dorsblot	0.1921**	0.0437	0.1107**	-0.0011	0.0845
tailblot	0.1642**	0.0515	0.0897**	0.0877**	0.0388
ventmot	0.0055	-0.0157	0.0372*	0.0301	-0.0456
genscut	0.1055**	0.0680**	0.0690**	0.0601	0.0349
genheadsc	0.1103**	0.0073**	0.0948**	0.0985**	0.0856*
ventrals	0.1160	0.0164	0.0601**	0.0370	0.0261
dorsrows	0.0604*	0.0318	0.0450*	0.0133	-0.0250
tailrows	0.0397	0.0480	-0.0071	0.0589	0.0322

Western females n=112

Character	Patristic distance	Geographic position	Vegtation	Monthly temperature	Monthly rainfall
genmorph	0.2135**	0.1486**	0.1387**	0.1489**	0.1262**
headshape	0.0139	0.1474**	0.0640*	0.1600**	0.1027
headsize	0.0397	0.1187*	0.0206	0.0845	0.0429
genpat	0.3150**	0.1264**	0.1196**	0.0750	0.1170**
genblot1	0.2408**	0.1019*	0.0862**	0.0595	0.1486**
genblot2	0.2002**	0.0715	0.0819**	0.0386	0.0620
genblot3	0.3238**	0.1789**	0.1540**	0.1191**	0.1847**
genblotw	0.1149*	0.0198	-0.0128	-0.0338	-0.0251
dorsblot	0.2484**	0.0639*	0.1115**	0.0900*	0.1083**
tailblot	0.1694**	0.0265	0.0555*	0.0258	-0.0286
ventmot	-0.0081	0.0337	0.0291	-0.0145	-0.0502
genscut	0.1258**	0.0125	0.0660*	0.0339	0.0664
genheadsc	0.1554**	0.0478	0.0601**	0.0825*	0.1306**
ventrals	0.1553**	-0.0246	0.0305	0.0292	-0.0091
dorsrows	0.0474	-0.0070	0.0598*	0.0222	0.0718
tailrows	0.0686*	0.0235	-0.0215	-0.0274	-0.0645

Great Basin and Pacific Coast males n=114

Character	Patristic distance	Geographic position	Vegetation	Monthly temperature	Monthly rainfall
genmorph	0.2947**	0.1143**	0.2049**	0.2216**	0.1152*
headshape	0.0425*	-0.0079	0.0066	0.1525**	-0.0495
headsize	0.0344	0.0006	0.0147	0.1248**	-0.0269
genpat	0.3990**	0.1891**	0.2447**	0.1597**	0.2712**
genblot1	0.2905**	0.0996**	0.1649**	0.0995**	0.1483**
genblot2	0.2614**	0.1443**	0.1851**	0.1469**	0.1946*
genblot3	0.3844**	0.2569**	0.2335**	0.1138**	0.2878**
genblotw	0.1949**	0.1048**	0.1100**	0.0689*	0.0704
dorsblot	0.3222**	0.0959**	0.1950**	0.0681**	0.2328**
tailblot	0.3076**	0.0833*	0.1122**	0.1541**	0.1039*
ventmot	-0.0231	0.0122	0.0716**	-0.0019	0.0234
genscut	0.1360**	0.0684	0.0953**	0.0691	-0.0157
genheadsc	0.1830**	0.0576	0.1275**	0.1522**	0.8919*
ventrals	0.1274**	0.0323	0.0917**	0.0555	0.0782
dorsrows	0.0631**	0.0216	0.0433	-0.0094	-0.0709
tailrows	0.0114	0.0267	0.0456	0.0229	-0.0354

Great Basin and Pacific Coast females n=102

Character	Patristic distance	Geographic position	Vegetation	Monthly temperature	Monthly rainfall
genmorph	0.2045**	0.1396*	0.1722**	0.1434*	0.1325*
headshape	0.0266	0.1417*	0.0640**	0.1972**	0.0957
headsize	0.0878**	0.1479*	0.0135	0.1320*	0.0525
genpat	0.3689**	0.1412**	0.1256**	0.0919	0.1371*
genblot1	0.2900**	0.1180*	0.0832**	0.0932	0.1828**
genblot2	0.2520**	0.0793	0.0962**	0.0614	0.0854
genblot3	0.3555**	0.1889**	0.1717**	0.1436**	0.2051**
genblotw	0.1305**	-0.0031	-0.0052	-0.0424	-0.0367
dorsblot	0.2381**	0.0749*	0.1175**	0.0947*	0.1346**
tailblot	0.2186**	0.0133	0.0418	0.0131	-0.0390
ventmot	0.0106	0.0646	0.0568*	-0.0110	-0.0541
genscut	0.1100**	-0.0150	0.1126**	-0.0180	0.0693
genheadsc	0.1578**	0.0470*	0.0778**	0.0643**	0.1138*
ventrals	0.2019**	0.0158	0.0801**	0.0966*	0.0282
dorsrows	-0.0013	-0.0438	0.0808**	-0.0483	-0.0733
tailrows	0.0962**	-0.0020	0.0380	-0.0692	-0.0721

Pacific Coast males n=72

Character	Patristic distance	Geographic position	Vegetation	Monthly temperature	Monthly rainfall
genmorph	0.0916**	-0.0196	0.1001**	0.0331	-0.0425
headshape	0.0041	-0.0245	-0.0260	0.1004	-0.0742
headsize	-0.0192	-0.0258	-0.0358	0.0570	-0.0564
genpat	0.0113**	0.0321	0.1277**	0.0136	0.0857
genblot1	0.0420*	-0.0224	0.0501	-0.0568	0.0078
genblot2	0.0163	0.0620	0.1145**	0.0362	0.0308
genblot3	0.2066**	0.1849**	0.1404**	0.1207**	0.1938**
genblotw	0.1592**	0.0537	0.0667*	-0.0349	0.0543
dorsblot	0.0946**	-0.0234	0.0767**	-0.0253	0.0707
tailblot	0.0532**	-0.0732	-0.0013	0.0034	0.0047
ventmot	-0.0082	-0.0857	0.0230	-0.0793	-0.0572
genscut	0.0526*	0.0029	0.0733*	-0.0101	-0.0408
genheadsc	0.1072**	0.0554	0.0780**	0.0571	0.0765
ventrals	0.0310	-0.0975	0.0687*	0.0082	0.0404
dorsrows	0.0239	-0.0065	0.0462	-0.0474	-0.0958
tailrows	-0.0083	-0.0178	-0.0155	-0.0099	-0.0515

Pacific Coast females n=70

Character	Patristic distance	Geographic position	Vegetation	Monthly temperature	Monthly rainfall
genmorph	0.0887**	0.0727	0.1109**	0.1021	0.0899
headshape	0.0019	0.1302*	0.0348	0.3094*	0.1135
headsize	0.0362	0.1456	-0.0111	0.1577	0.1140
genpat	0.1234**	0.0669	0.0288	0.0653	0.0708
genblot1	0.0870**	0.0637	0.0311	0.1180	0.1659
genblot2	0.0783**	0.0021	0.0061	0.0219	0.0036
genblot3	0.0953**	0.1137	0.1178**	0.1302	0.1540*
genblotw	0.1091**	0.0253	-0.0314	-0.0799	-0.0311
dorsblot	0.0324	0.0542	-0.0087	0.0733	0.1054
tailblot	0.0427	-0.0127	-0.0223	-0.026	-0.0597
ventmot	0.0484	0.0549	0.0422	-0.0364	-0.0681
genscut	0.0480	-0.0809	0.0883*	-0.0588	0.0505
genheadsc	0.0962**	0.0546	0.0516	0.0587	0.0608
ventrals	-0.0120	-0.0925	0.0404	0.1252	-0.0523
dorsrows	-0.0077	0.0877	0.0682	-0.0778	0.1026
tailrows	0.0576*	-0.0062	0.0946**	-0.0811	-0.0913

Appendix IX. Venom samples used in isoelectric focusing

The field references (Ref) are those of C. E. Pook. The second reference includes personal credits for the sample, institution voucher/accession number, or captivity reference code. UTEP = University of Texas El Paso; ASDM = Arizona-Sonora Desert Museum; KA = Kyle Ashton; MC = Mike Cardwell; CAS = California Academy of Sciences; WH = Bill Hayes; LS = Lee Simons; UWB = University of Wales, Bangor; WW = W. Wüster. Numbers in square brackets indicate classification of venom profiles, i.e. idential profiles have the same number.

Ref	Taxon	Locality / [venom type code]		Grid Reference	Ref 2
064	*concolor*	Flaming Gorge Res., Sweetwater Co., WY	[1]	41.134 109.528	KA
065	*concolor*	Flaming Gorge Res., Sweetwater Co., WY	[1]	41.134 109.528	KA
066	*concolor*	Flaming Gorge Res., Sweetwater Co., WY	[1]	41.134 109.528	KA
068	*concolor*	Flaming Gorge Res., Sweetwater Co., WY	[1]	41.134 109.528	KA
069	*concolor*	Flaming Gorge Res., Sweetwater Co., WY	[1]	41.134 109.528	KA
071	*concolor*	Flaming Gorge Res., Sweetwater Co., WY	[1]	41.134 109.528	KA
072	*concolor*	Flaming Gorge Res., Sweetwater Co., WY	[1]	41.134 109.528	KA
047	*cerberus*	Mt. Lemmon, Pima Co., AZ	[2]	32.450 111.050	ASDM91076
127	*cerberus*	Stockton Pass Wash, Graham Co., AZ	[3]	32.592 109.850	
133	*cerberus*	Hwy 89, nr. Payson, Gila Co., AZ	[4]	34.284 111.334	
021	*lutosus*	Washington Co., UT	[5]	37.250 113.500	MC
124	*lutosus*	Red Butte, Salt Lake Co., UT	[6]	40.500 112.000	
125	*lutosus*	Red Butte, Salt Lake Co., UT	[7]	40.500 112.000	
126	*lutosus*	Red Butte, Salt Lake Co., UT	[8]	40.500 112.000	
131	*nuntius*	Coconino Co., AZ	[9]	35.192 111.374	UWB
132	*nuntius*	Coconino Co., AZ	[9]	35.118 111.031	UWB
129	*viridis*	Old highway 666, Apache Co., AZ	[10]	34.191 109.343	
043	*lutosus*	Page area, AZ	[22]	37.000 111.500	
130	*viridis*	Old highway 666, Apache Co., AZ	[10]	34.191 109.343	
045	*abyssus*	Grand Canyon, Coconino Co., AZ	[11]	36.000 113.500	ASDM95069
048	*viridis*	State Hwy 9, Dona Ana Co., NM	[12]	31.817 106.734	UTEP17625
085	*viridis*	State Hwy 9, Dona Ana Co., NM	[13]	31.817 106.734	UTEP - MM26
086	*viridis*	State Hwy 9, Dona Ana Co., NM	[14]	31.817 106.734	UTEP - MM27
044	*viridis*	Delaware R, S of Carlsbad, Eddy Co., NM	[15]	32.000 104.000	ASDM92290
046	*viridis*	Fort Bliss, El Paso, TX	[16]	31.900 106.250	ASDM92302
062	*viridis*	Horse Creek, Laramie Co., WY	[17]	41.400 105.200	KA
001	*oreganus*	Sniveley Jctn, Tehama Co., CA	[18]	40.317 122.234	LS
004	*oreganus*	Sniveley Jctn, Tehama Co., CA	[19]	40.317 122.234	LS
025	*oreganus*	Pullman,Whitman Co., WA	[20]	46.700 177.000	WH CVO13
026	*oreganus*	Summit Rd, Santa Cruz Co., CA	[21]	37.000 122.000	WH CVO03
030	*helleri*	San Jacinto Mt., Riverside Co., CA	[30]	34.000 117.000	WH CVO20
087	*oreganus*	near Yreka, Siskiyou Co., CA	[23]	42.000 122.617	LS
088	*oreganus*	border with Oregon, Siskiyou Co., CA	[23]	41.750 122.617	LS
089	*oreganus*	Tarantula Gulch, Shasta Co., CA	[24]	41.167 122.117	LS
090	*oreganus*	I5 betwn Yreka & Weed, Siskiyou Co., CA	[25]	41.750 122.617	LS
118	*oreganus*	Corral Hollow, Alameda Co., CA	[26]	37.917 122.250	CAS-RL001
023	*he/or*	Paso Robles, San Luis Obispo Co., CA	[27]	35.634 120.717	MC
022	*helleri*	San Bernardino Co., CA	[28]	34.140 117.190	MC
005	*helleri*	Loma Linda, San Bernardino Co., CA	[29]	34.100 117.150	WH
007	*helleri*	San Jacinto Mt., Riverside Co., CA	[30]	34.000 117.000	WH CVH65
009	*helleri*	San Jacinto Mt., Riverside Co., CA	[31]	34.000 117.000	WH CVH91
027	*helleri*	San Jacinto Mt., Riverside Co., CA	[32]	34.000 117.000	WH CVH93
032	*helleri*	San Jacinto Mt., Riverside Co., CA	[33]	34.000 117.000	WH CVH81
034	*helleri*	San Jacinto Mt., Riverside Co., CA	[33]	34.000 117.000	WH CVH83
031	*helleri*	San Jacinto Mt., Riverside Co., CA	[33]	34.000 117.000	WH CVH22
035	*helleri*	San Jacinto Mt., Riverside Co., CA	[33]	34.000 117.000	WH CVH87
033	*helleri*	San Jacinto Mt., Riverside Co., CA	[34]	34.000 117.000	WH CVH79
036	*helleri*	San Jacinto Mt., Riverside Co., CA	[36]	34.000 117.000	WH CVH85
038	*helleri*	San Jacinto Mt., Riverside Co., CA	[37]	34.000 117.000	WH CVH75
040	*helleri*	San Bernardino Co., CA	[28]	34.100 117.150	WH CVH11
119	*helleri*	Otay Mesa, San Diego Co., CA	[38]	32.573 116.944	
120	*helleri*	Otay Mesa, San Diego Co., CA	[39]	32.573 116.944	
121	*helleri*	Otay Mesa, San Diego Co., CA	[39]	32.573 116.944	
122	*helleri*	Otay Mesa, San Diego Co., CA	[40]	32.573 116.944	
123	*helleri*	Otay Mesa, San Diego Co., CA	[40]	32.573 116.944	

Appendix X. Protocols for isoelectric focusing

Table X.1. Isolectric focusing of snake venom proteins

EQUIPMENT

pH gels 3.5-9.5 (Ampholine PAG™) plates

LKB flat-bed electrofocusing apparatus Multiphor™ II 2117 (Pharmacia, Herts., UK)

SOLUTIONS

Fixing solution:

29g Trichloroacetic acid and 8.5 g Sulphosalicylic acid dissolved in distilled water and made up to 250 ml.

Destaining solution: 500 ml Ethanol (or industrial methylated spirit) and 160 ml Acetic acid, made up to 2 litres with distilled water.

Staining solution: Dissolve 0.46g Coomassie blue R-250 in destaining solution. Filter before use.

Preserving solution: 25 ml glycerol made up to 250 ml with destaining solution.

Note: Electrophoresis conditions may need to be optimised according to the type of venom being used.

REHYDRATION OF VENOMS

1 Dissolve a sample of crystalline venom in ultra-pure water to make up to a concentration of 10 mg/ml soluble protein.

2 Centrifuge at 13 000 rpm for 30 seconds

APPLICATION OF SAMPLES TO AMPHOLINE PAG™ GEL

1 Add 5 µl of each supernatant to the Multiphor™ II to the wells laid across the centre of the gel.

2 At intervals, add 5 µl of pH marker

3 Prepare the electrode strips

- soak the anode strip in 1M orthophosphoric acid
- soak the cathode strip in 1M sodium hydroxide

Blot to remove excess liquid, and apply to the top and bottom of the gel respectively about 5 mm away from the edges

4 Allow the system to adjust to 5 °C for 15 minutes prior to electrophoresis.

The system should also be maintained at 5 °C for the duration of electrophoresis.

ELECTROPHORESIS, FIXING AND STAINING

1 Run the samples for 80 minutes at 1500 V, 50mA (25mA if gel halved), 30W (15W if gel halved). Carefully remove the electrodes after the run

2 **Fixing:** To fix the proteins in the gel and precipitate out the ampholytes, introduce to a bath of fixing solution for 1 hour

3 **Staining:** Wash the gel by soaking in destaining solution for 5 minutes. To stain, soak in Coomassie blue R-250 (solution preheated to 60 °C before use) for 10 minutes

4 **Destaining:** Wash several times in destaining solution until the background is clear

5 **Preserving:** Soak in preserving solution overnight. Drain off excess liquid, cover with film, and allow to dry at room temperature.

Table X.2. Isoelectric focusing calibration markers: Broad pI kit (pH3-10) (Pharmacia Biotech).

Protein	**pI**
Amyloglucosidase	3.50
Methyl red (dye)	3.75
Soybean trypsin inhibitor	4.55
β-lactoglobulin A	5.20
Bovine carbonic anhydrase B	5.85
Human carbonic anhydrase B	6.55
Horse myoglobin - acidic	6.85
Horse myoglobin - basic	7.35
Lentil lectin - acidic	8.15
Lentil lectin - middle	8.45
Lentil lectin - basic	8.65
Trypsinogen	9.30