

Molecular and historical evidence for the introduction of clouded salamanders (genus *Aneides*) to Vancouver Island, British Columbia, Canada, from California

Todd R. Jackman

Abstract: Genetic evidence shows that Vancouver Island populations of clouded salamanders (genus *Aneides*) are nearly identical with some California populations, in both allozymes and mitochondrial DNA. Historical evidence suggests that salamanders were introduced to Vancouver Island during the nineteenth century. They were probably included in shipments of tan oak bark from California. Tan oak bark was used extensively in the tanning of leather at that time. The introduction to Vancouver Island provides an opportunity to study environmental effects in a species that can not be easily studied on a short-term basis. The California and Canadian populations differ strikingly from Oregon populations of clouded salamanders and are described as a new species, *Aneides vagrans*.

Résumé : Des caractéristiques génétiques indiquent que les populations de Salamandres pommelées (*Aneides*) de l'Île de Vancouver sont presque identiques aux populations de la Californie, à la fois par leurs allozymes et par leur ADN mitochondrial. Des preuves historiques indiquent que les salamandres ont été introduites dans l'île au cours du dix-neuvième siècle. Elles sont probablement venues de Californie avec des chargements d'écorce à tan, très utilisée à cette époque pour le tannage du cuir. L'introduction des populations dans l'île de Vancouver donne lieu à la possibilité d'étudier les effets de l'environnement sur une espèce qui ne peut être facilement étudiée à court terme. Les populations de Californie et du Canada diffèrent fortement des populations d'*Aneides* de l'Oregon et sont décrites ici comme appartenant à une nouvelle espèce, *Aneides vagrans*.

[Traduit par la Rédaction]

Introduction

The clouded salamander, *Aneides ferreus*, is found in the coastal forests of northwestern California, throughout western Oregon, on Vancouver Island and its neighboring small islands, and in a small area of the coastal mainland in British Columbia (Fig. 1; Stebbins 1985). This distribution is unusual because of the absence of the species from the state of Washington. Every other species of plant and animal that occurs in the forests of Oregon and Vancouver Island also occurs in the state of Washington. The absence of the salamanders from Washington has been explained as the result of only partial coverage of Vancouver Island by glaciers during the ice age, providing a refuge for the salamanders not available in Washington (Peabody and Savage 1958). Clouded salamanders live primarily under the bark of trees but can also be found under rocks on talus slopes in some areas. They occur in sympatry with the other two western species of *Aneides* in northern California.

In published accounts of chromosomal variation in *A. ferreus*, Sessions and Kezer found that Vancouver Island individuals resembled populations from northern California in their karyotype (Kezer and Sessions 1979; Sessions and Kezer 1987). They also found that Oregon populations differed from the other two in many respects. They suggested that *A. ferreus* may have been introduced to Vancouver Island from California. This was the first suggestion that *A. ferreus* consisted of two genetically distinct groups of populations. I use the nomenclature of Sessions and Kezer (1987) when referring to the two groups of salamanders. *Aneides ferreus* I refers to the Oregon karyotype (populations 4–14, C–F; Figs. 1 and 2) and *A. ferreus* II refers to all other populations (1–3 and 15–25, A, B, and G–M; Figs. 1 and 2).

Beatty (1983) documented morphometric variation throughout the range of *A. ferreus*. The populations showed a large amount of within-population variation, but few individual characters varied consistently between populations. However, in a multivariate morphometric plot using all of the morphometric characters, most populations could be distinguished statistically from one another (Fig. 3). Only three populations were indistinguishable. These populations were from northern California and Vancouver Island, again suggesting a link between the two places.

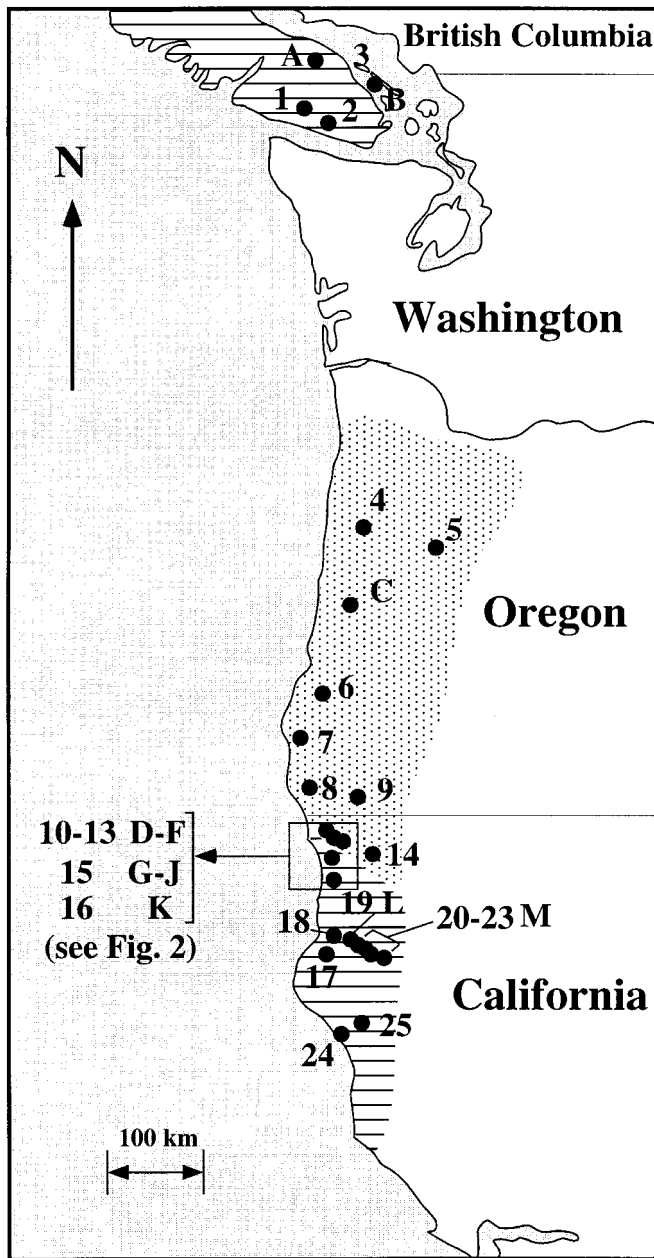
I present allozyme and mitochondrial DNA evidence that there are two species of clouded salamanders. Furthermore, I present genetic and historical evidence that the California

Received September 19, 1997. Accepted March 10, 1998.

T.R. Jackman.¹ Museum of Vertebrate Zoology and Department of Integrative Biology, 3101 Valley Life Sciences Building, University of California, Berkeley, CA 94720, U.S.A.

¹Present address: Department of Biology, Campus Box 1137, Washington University, St. Louis, MO 63130–4899, U.S.A.

Fig. 1. Range and sampling localities for the large-scale allozyme survey (populations 1–25) and mitochondrial analysis (A–M). The stippled area corresponds to the range of chromosomal variants designated as *Aneides ferreus* I (Sessions and Kezer 1987) and the hatched area corresponds to the range of chromosomal variants designated as *Aneides ferreus* II. Exact localities are available from the NRC depository.²



species of clouded salamanders was introduced to Vancouver Island.

The first report of *A. ferreus* in British Columbia was in 1906 from one of the many small islands off the coast of Vancouver Island (Slevin 1928). Tan oak (*Lithocarpus*

densiflorus) bark was shipped in large quantities from California to Vancouver Island during the latter half of the nineteenth century for use in the tanning of leather goods. The first leather tannery opened on Vancouver Island in 1842. From the descriptions of the process of stripping and transporting the bark (Jepson 1911) it seems likely that clouded salamanders were included with shipments of the bark. I present historical evidence that salamanders were introduced to Vancouver Island from California sometime between 1842 and 1906.

Methods and materials

Allozymes

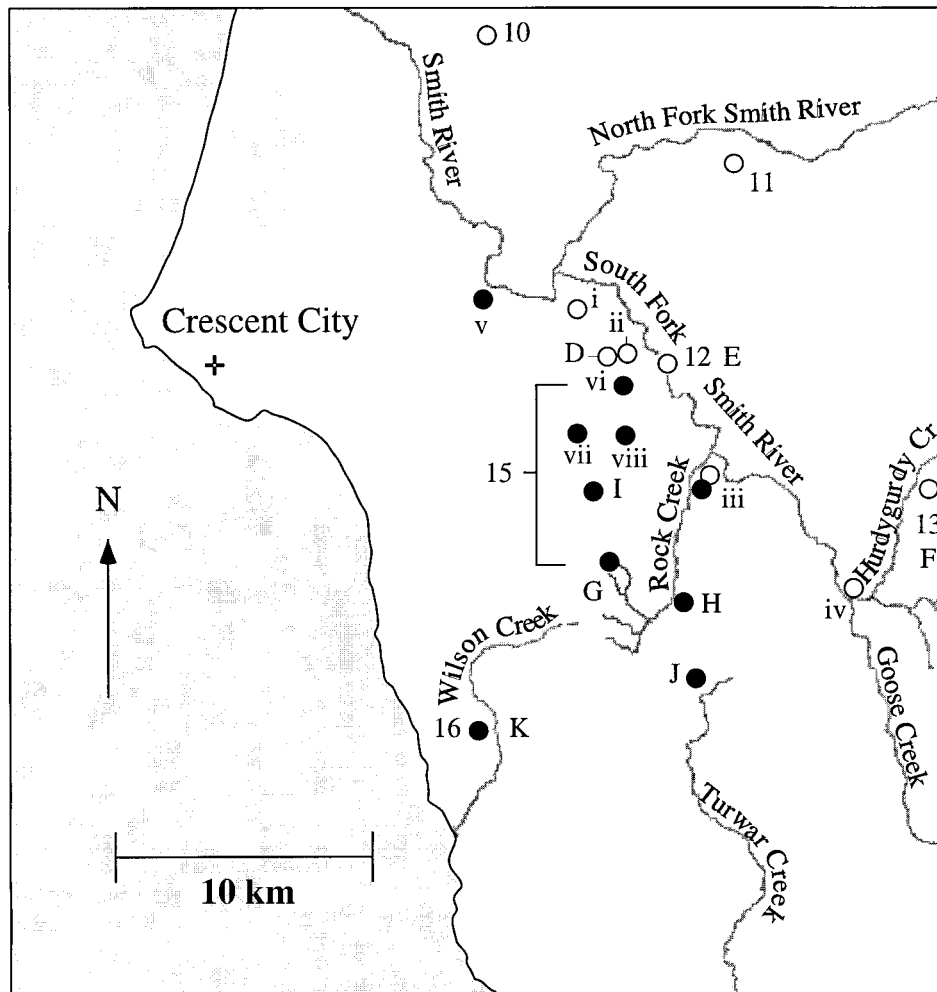
I examined samples of *A. ferreus* collected from populations occurring throughout the range of the species (Fig. 1). My main analysis utilizes 25 samples ranging in size from 4 to 31 specimens, but I have obtained useful information from smaller samples taken from geographically important populations (exact localities (Appendices 2 and 3) have been deposited in the National Research Council of Canada (NRC) Depository of Unpublished Data).² In particular, samples of one or two individuals were used to pinpoint a genetic break between populations along the South Fork of the Smith River near the Oregon border in Del Norte County, California. For comparisons between all populations, samples of one to three individuals in the zone of contact were either excluded or grouped with geographically close larger populations. In this region many populations were sampled on a small scale (Fig. 2). Starch-gel electrophoresis was used to examine protein variation in the samples, following the methods of Wake and Yanev (1986). Freshly sacrificed specimens were dissected and tissue samples (liver and intestine) were stored at -76°C until used. Carcasses were preserved as voucher specimens in the collections of the Museum of Vertebrate Zoology, University of California, Berkeley. Aqueous mixed homogenates of the tissues were assayed using standard horizontal starch-gel electrophoresis and histochemical staining procedures (Selander et al. 1971; Ayala et al. 1972; Harris and Hopkinson 1976; Murphy et al. 1996). Variants are designated alphabetically, "a" being the fastest migrant. Polymorphism is based on all observed variants, and heterozygotes were recorded from direct counts. Buffer conditions used were the same as those listed for Jackman and Wake (1994) based on Selander et al. (1971), except for Pep-S (LiOH, pH 8.2), Mdhp, and Acp (Tris-citrate II, pH 8.0).

DNA preparation, amplification, and sequencing

Samples from *Aneides flavipunctatus*, *A. ferreus* I (C–F; Figs. 1 and 2), and *A. ferreus* II (A, B, and G–M; Figs. 1 and 2) were used in the DNA analysis (sequences are available on GenBank). Genomic DNA was extracted from ethanol-preserved liver samples using the Qiagen QIAamp tissue kit. Amplification of genomic DNA was conducted using the polymerase chain reaction (PCR) with denaturation at 94°C for 35 s, annealing at 48°C for 35 s, and extension at 72°C for 150 s, with 4 s added to the extension per cycle, for 30 cycles. Primers L4437b (Macey et al. 1997b) and H5617a (Macey et al. 1997a) were used to amplify the entire mitochondrial NADH dehydrogenase subunit 2 (ND2) gene and the tryptophan transfer RNA. Amplified products were purified on 1.5% Nusieve GTG agarose gels and reamplified under similar conditions. Reamplified double-stranded products were purified on 2.5% acrylamide gels (Maniatis et al. 1982). Template DNA was

²Copies of Appendices 2 and 3, listing localities of the populations shown in Figs. 1 and 2 and Nei's (1972, 1978) genetic distances, respectively, may be purchased from the Depository of Unpublished Data, CISTI, National Research Council of Canada, Ottawa, ON K1A 0S2, Canada.

Fig. 2. Populations sampled in Del Norte County, California, in the area of contact between the two major groups of *Aneides*. Open circles indicate populations of *Aneides ferreus* I and solid circles indicate populations of *Aneides ferreus* II. Arabic numbers denote populations used for the large-scale allozyme analysis; lower case roman numerals denote small populations sampled, and letters denote locations of individuals from the mitochondrial analysis. Exact localities are listed the NRC depository (see footnote 2).

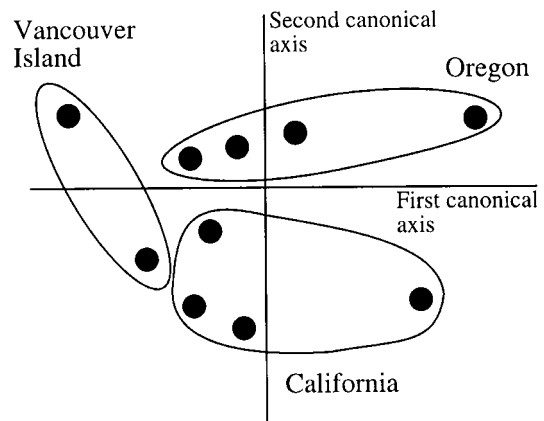


eluted from acrylamide passively with Maniatis elution buffer (Maniatis et al. 1982). Cycle-sequencing reactions were run using the Promega fmol DNA sequencing system with denaturation at 95°C for 35 s, annealing at 60°C for 35 s, and extension at 70°C for 1 min for 30 cycles. The primer H5617a was used to sequence the 3' end of ND2 and the tryptophan transfer RNA, for a total of 554 bases. Sequencing reactions were run on Long Ranger sequencing gels for 4–12 h at 38–42°C. All PCR reactions included negative controls to guard against contamination of reagents with DNA.

Data analysis

Genetic distances were calculated using the methods of Nei (1972, 1978) with the BIOSYS-1 program (Swofford and Selander 1981). Heterozygosities were also calculated using BIOSYS-1. Nei's genetic distance matrix was analyzed using the Fitch and Margoliash (1967) method with the software package PHYLIP (version 3.5; Felsenstein 1993). These methods were chosen because they incorporate variation in branch length when the tree is constructed. The global rearrangement and jumble options were employed when using the Fitch program in PHYLIP to avoid finding suboptimal arrangements associated with the order in which taxa are analyzed in the program (Felsenstein 1993).

Fig. 3. Morphometric variation (modified from Beatty 1983). Population means from a multivariate morphometric survey of 10 characters throughout the range of *A. ferreus* are shown.



Mitochondrial DNA sequences were analyzed using PAUP 3.1 (Swofford 1992). Base positions were treated as characters with up to four unordered states for each character. One sequence from

A. flavipunctatus was used as an outgroup sequence. Bootstrap values were calculated using PAUP 3.1 with 1000 replicates and branch and bound searches. Decay index ("branch support" of Bremer 1994) values were also calculated using PAUP 3.1 by examining all trees within 13 steps of the most parsimonious tree. Percent difference between sequences was calculated using PAUP. Kimura's two-parameter distances were also calculated using observed transition to transversion ratios with the DNA distance program in PHYLIP.

Historical evidence

Most of the historical research was done using the facilities of the Bancroft Library at the University of California, Berkeley. Bauer (1957) contains a detailed list of all references to the tanning industry in California before 1900 in all of the newspapers along the California coast. All references concerning newspaper accounts and statistics contained therein were obtained through the bibliography of Bauer (1957).

Results

The allozyme variation is summarized in Table 1. The degree of variation seen within *A. ferreus* populations is similar to levels in other western plethodontid salamanders such as *Plethodon dunni* and *Plethodon larselli* (Highton and Larson 1979), but less than that seen in *Ensatina escholtzii* (Wake and Yanev 1986) or *A. flavipunctatus* (Larson 1980). For example, heterozygosities were between 0 and 8%. Most populations had heterozygosities of less than 5%. Within *Ensatina* and *A. flavipunctatus*, heterozygosities are as high as 20% (Larson 1980; Wake and Yanev 1986).

Nei's unbiased genetic distances up to 0.54 were observed between members of the chromosomal types (data are available from the NRC depository; see footnote 2). Genetic distances are larger within northern California than between Vancouver Island and some northern California populations. The largest genetic distance within *A. ferreus* I is 0.175; within *A. ferreus* II the largest genetic distance is 0.1. The genetic distances were analyzed using the method of Fitch and Margoliash (1967) (Fig. 4). In Fig. 4, a large genetic break between the two chromosomal types is evident, as well as relatively small distances within each group. The neighbor-joining method (Saitou and Nei 1987) produces a tree with similar branch lengths and topology. Six fixed differences diagnose the two groups (Aat-2, Idh-1, G3pdh, La, Gtdh, and Ldh-1).

In many cases, allelic variants are geographically localized. For example, Idh-2 (a) is fixed in the northern cascade population (5) and is also found in populations 4, 6, and 9. Ldh-1 (b) is only found as a rare variant in the southwest portion of the range of *A. ferreus* I in populations 10, 11, 13, and 14. Gpi (b) is present only in *A. ferreus* I in all populations except 5, 7, and 9. Adh-1 (a) is found on Vancouver Island (populations 1–3), three populations east of Eureka, California (17, 19, and 20), and in the northernmost California population of *A. ferreus* II (15). Three individuals of *A. ferreus* I from three different populations are heterozygous for G3pdh, one of the six loci that diagnose the two groups of *A. ferreus*. One is near the zone of contact between the two groups and may be the result of introgression. The other two are from central and northern Oregon (popu-

lations 4 and 5). Mdhp (a) is fixed in the southernmost populations of *A. ferreus* II. Mdhp (b) is a variant present in the northwestern California portion of the range of *A. ferreus* II (populations 15–20), and is also found on Thetis Island, off the coast of Vancouver Island (population 3).

Contact between *A. ferreus* I and *A. ferreus* II

Sampling was performed on a smaller scale in the area east of Crescent City, Del Norte County, California, where the chromosome types come in contact. Figure 2 shows the localities of samples taken in Del Norte County, and the designation of populations as either *ferreus* I or *ferreus* II. For the large-scale allozyme survey, the six northernmost *ferreus* II populations were lumped together as a single population (15; Figs. 1 and 2).

Of the 91 individuals found in the contact zone, 11 (12%) had introgressed alleles. Introgressed alleles are exceptions to the six fixed differences that define the two groups. We found no F₁ hybrids. Also, no backcrossed (i.e., F₂) individuals were identified. Introgression appears to be the result of rare past hybridization events. The degree of introgression is far less than that which has been observed between some parapatric species of the *Plethodon glutinosus* complex (Highton 1989). Five of the six fixed loci had at least one introgressed allele. In total, five individuals of *ferreus* I were found with *ferreus* II alleles and six individuals of *ferreus* II were found with *ferreus* I alleles. All of the individuals of *ferreus* II with introgressed alleles were males. However, it is unclear whether this was due to the small sample size or some other factors. Along Rock Creek, individuals identifiable as *ferreus* I and *ferreus* II (with no introgression) can be found in sympatry. Hybridization is patchy and limited in scope.

Mitochondrial DNA

For the mitochondrial ND2 and tRNA data, there were 13 ingroup sequences (i.e., *A. ferreus*) and one outgroup sequence (*A. flavipunctatus*). Of the 142 variable sites in the sequence, 59 were potentially phylogenetically informative; that is, they were sites where two states are shared by at least two individuals and at least two other states are shared by two different taxa. Within the 13 ingroup sequences there are 71 variable characters and 56 potentially phylogenetically informative characters. Two pairs of the sequences from Del Norte County were identical (G and H, I and K). Of the 43 unambiguous character changes in the ingroup, 7 represent transversional changes and 36 represent transitions. The observed transition to transversion ratio is therefore 5:1, similar to the rate observed in other vertebrates (Brown 1985; Moritz et al. 1992).

Within the ingroup, uncorrected sequence difference between individuals varied from 0 to 10% (Table 2). Differences ranged from 0.2 to 2.9% within *A. ferreus* I and from 0 to 4.2% within *A. ferreus* II. Kimura's two-parameter distances are less than 0.1% larger within each chromosomal type, and less than 1% larger than uncorrected differences between the two groups.

A phylogenetic analysis of all 14 ND2 and tRNA sequences produced one most parsimonious tree (Fig. 5) 170 steps long and with a consistency index of 0.75 (excluding

Table 1. Gene frequencies; population gene frequencies are categorized into fixed and polymorphic, with the frequencies of alleles in polymorphic populations indicated to two digits.

Protein	EC No.	Fixed populations	Polymorphic populations
Pgdh	1.1.1.44	d (1–3, 12, 14–26), c (5, 9)	4 (a = 0.04, c = 0.96), 6 (c = 0.86, d = 0.14), 7 (c = 0.90, d = 0.10), 8 (c = 0.93, d = 0.07), 10 (b = 0.13, d = 0.83, e = 0.04), 11 (d = 0.95, e = 0.05), 13 (c = 0.25, d = 0.75)
Idh-1	1.1.1.42	a (4–14), b (1–3, 17–19, 24, 25), c (21–23), d (26)	15 (b = 0.16, c = 0.84), 16 (b = 0.33, c = 0.67), 20 (b = 0.15, c = 0.85)
Idh-2	1.1.1.42	b (1–3, 6–8, 10–24), a (5), c (26)	4 (a = 0.89, b = 0.11), 6 (a = 0.50, b = 0.50), 9 (a = 0.38, b = 0.62)
Acoh-1	4.2.1.3	c (1–8, 11, 12, 14, 16–25)	9 (c = 0.88, d = 0.12), 10 (c = 0.83, d = 0.17), 15 (a = 0.08, c = 0.92), 26 (c = 0.50, e = 0.50)
Acoh-2	4.2.1.3	a (1–13, 15–22, 24–26)	14 (a = 0.75, b = 0.25), 23 (a = 0.90, b = 0.10)
Ldh-1	1.1.1.27	a (1–3, 15–25), c (4, 5, 7–9, 12), e (26)	6 (c = 0.93, d = 0.07), 10 (b = 0.04, c = 0.96), 11 (b = 0.15, c = 0.85), 13 (b = 0.08, c = 0.92), 14 (b = 0.25, c = 0.75)
Ldh-2	1.1.1.27	a (1–8, 10–26)	9 (a = 0.25, b = 0.75)
Gpi	5.3.1.9	c (1–3, 7, 9, 15–25), b (4, 11–14), d (5), e (26)	6 (a = 0.17, b = 0.33, c = 0.50), 8 (b = 0.93, c = 0.07), 10 (b = 0.46, c = 0.54)
Ada-1	3.5.4.4	a (16), b (1–5, 7–14, 16–18, 20–25), d (26)	6 (b = 0.86, c = 0.14), 15 (a = 0.98, b = 0.02), 19 (a = 0.36, b = 0.64)
Ada-2	3.5.4.4	c (1–5, 7–9, 11–13, 15–21, 23–25), f (26)	6 (b = 0.14, c = 0.86), 10 (b = 0.04, c = 0.96), 14 (a = 0.13, c = 0.75, e = 0.12), 22 (c = 0.75, d = 0.25)
Adh-1	1.1.1.1	b (1, 4–14, 16, 18, 21–25), a (1)	2 (a = 0.83, b = 0.17), 3 (a = 0.83, b = 0.17), 15 (a = 0.10, b = 0.90), 17 (a = 0.14, b = 0.86), 19 (a = 0.64, b = 0.36), 20 (a = 0.12, b = 0.88)
Adh-2	1.1.1.1	a (2–25), c (26)	1 (a = 0.94, b = 0.06)
Mdh	1.1.1.37	a (1–3, 5–8, 10–25)	4 (a = 0.98, b = 0.02), 9 (a = 0.75, b = 0.25)
G3pdh	1.1.1.8	b (1–3, 16–25), a (5, 7–14)	4 (a = 0.96, b = 0.04), 6 (a = 0.93, b = 0.07), 10 (a = 0.96, b = 0.04), 15 (a = 0.03, b = 0.97)
Iddh	1.1.1.14	a (1–25), b (26)	None
Mpi	5.3.1.8	a (1–25), b (26)	None
Pgm	5.4.2.2	c (1–8, 10, 12, 15–25), a (26)	9 (a = 0.62, c = 0.38), 11 (b = 0.2, c = 0.8), 13 (b = 0.08, c = 0.92), 14 (c = 0.75, d = 0.25)
Aat-1	2.6.1.1	a (1–25), b (26)	None
Aat-2	2.6.1.1	a (1–3, 5–25), b (4–14), c (26)	None
Pep-B	3.4.11.4	c (1–3, 5, 10–12, 15, 17–19, 24, 25), b (4), d (26)	6 (b = 0.33, c = 0.67), 7 (b = 0.14, c = 0.86), 8 (b = 0.12, c = 0.88), 9 (b = 0.25, c = 0.75), 13 (b = 0.25, c = 0.75), 14 (b = 0.25, c = 0.75), 16 (b = 0.08, c = 0.92), 20 (a = 0.15, c = 0.85), 21 (a = 0.75, c = 0.25), 22 (a = 0.62, c = 0.38), 23 (a = 0.10, c = 0.90)
Pep-S	3.4.11.4	c (1–3, 15–26), b (4–7, 9–14)	8 (a = 0.03, b = 0.97)
G6pdh	1.1.1.49	b (1, 4–23), a (2, 3, 24), c (25), d (26)	None
Hadh	1.1.99.6	a (1–26)	None
Mdhp	1.1.1.40	c (1, 2, 4–14, 21–23), a (24, 25), d (26)	3 (b = 0.08, c = 0.92), 15 (b = 0.24, c = 0.76), 16 (b = 0.17, c = 0.83), 17 (b = 0.88, c = 0.12), 18 (b = 0.75, c = 0.25), 19 (b = 0.71, c = 0.29), 20 (b = 0.08, c = 0.92)
Acp	3.1.3.2	a (1–6, 8–25), c (26)	7 (a = 0.86, b = 0.14)
Gp-1	Non-specific	a (1–26)	None
Gtdh	1.4.1.2	a (1–3, 16–24), b (4–9, 12–14, 26)	10 (a = 0.04, b = 0.96), 11 (a = 0.05, b = 0.95), 15 (a = 0.94, b = 0.06), 25 (a = 0.90, c = 0.10)

Note: The population numbers correspond to the localities on Figs. 1 and 2.

uninformative characters). Both chromosomal groups were well supported in the analysis, as indicated by both bootstrap and decay index values. Sixteen characters change unambiguously along the branch leading to *A. ferreus* II, 13 transitions and 3 transversions. Eleven characters change along the branch leading to *A. ferreus* I, 9 transitions and 2 transversions.

Three unambiguous changes (one transversion and two transitions) characterize the clade that includes the Vancouver Island and Humboldt County, California, individuals. These populations also share a high frequency of the Adh-1 (a) allele (see the allozyme results). The degree of mitochondrial differentiation between the Vancouver Island and Humboldt County individuals (1.3–1.5%) was only

Fig. 4. Tree constructed using the method of Fitch and Margoliash (1967) on Nei's (1978) genetic distances. Branches are proportional to the reconstructed distances. Numbers correspond to the populations identified in Figs. 1 and 2. The outgroup (*Aneides lugubris*) is not shown. The six fixed allozyme loci between the two groups are shown as changing along a particular branch (indicated by a tick mark). In cases where *A. ferreus* I, *A. ferreus* II, and the outgroup all have unique allelic states, no tick marks are indicated (Aat-2, Idh-1).

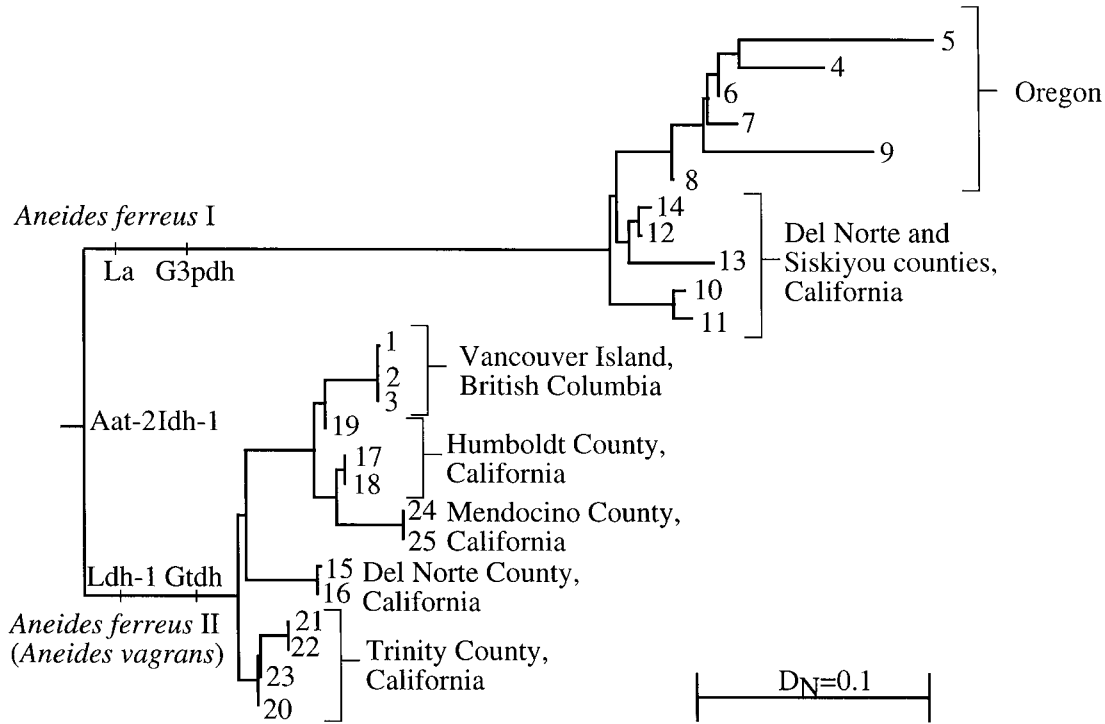


Table 2. Sequence differences in mtDNA among *Aneides ferreus* populations (A–M) and between *A. ferreus* and *A. flavipunctatus*.

	A	B	C	D	E	F	G	H	I	J	K	L	M	<i>A. flavipunctatus</i>
A	—	0.009	0.085	0.093	0.089	0.085	0.025	0.025	0.024	0.027	0.024	0.015	0.031	0.195
B	5	—	0.091	0.100	0.094	0.091	0.020	0.020	0.018	0.022	0.018	0.013	0.040	0.191
C	47	50	—	0.024	0.029	0.025	0.087	0.087	0.085	0.085	0.085	0.079	0.080	0.182
D	39	42	10	—	0.002	0.002	0.096	0.096	0.093	0.091	0.093	0.085	0.089	0.180
E	49	52	16	1	—	0.004	0.091	0.091	0.089	0.089	0.089	0.083	0.087	0.180
F	47	50	14	1	2	—	0.087	0.087	0.085	0.085	0.085	0.079	0.083	0.180
G	14	11	48	40	50	48	—	0.000	0.002	0.005	0.002	0.021	0.040	0.196
H	14	11	48	40	50	48	0	—	0.002	0.005	0.002	0.021	0.040	0.196
I	13	10	47	39	49	47	1	1	—	0.004	0.000	0.019	0.038	0.195
J	15	12	47	38	49	47	3	3	2	—	0.004	0.023	0.042	0.195
K	13	10	47	39	49	47	1	1	0	2	—	0.019	0.038	0.195
L	8	7	42	34	44	42	11	11	10	12	10	—	0.039	0.186
M	17	22	44	37	48	46	22	22	21	23	21	21	—	0.198
<i>A. flavipunctatus</i>	107	105	100	75	99	99	108	108	107	107	107	99	109	—

Note: The pairwise percent differences between sequences of mtDNA are shown above the diagonal and the number of differences below.

slightly higher than that observed between populations in Del Norte County on a small geographic scale (up to 0.5% divergence across 10 km).

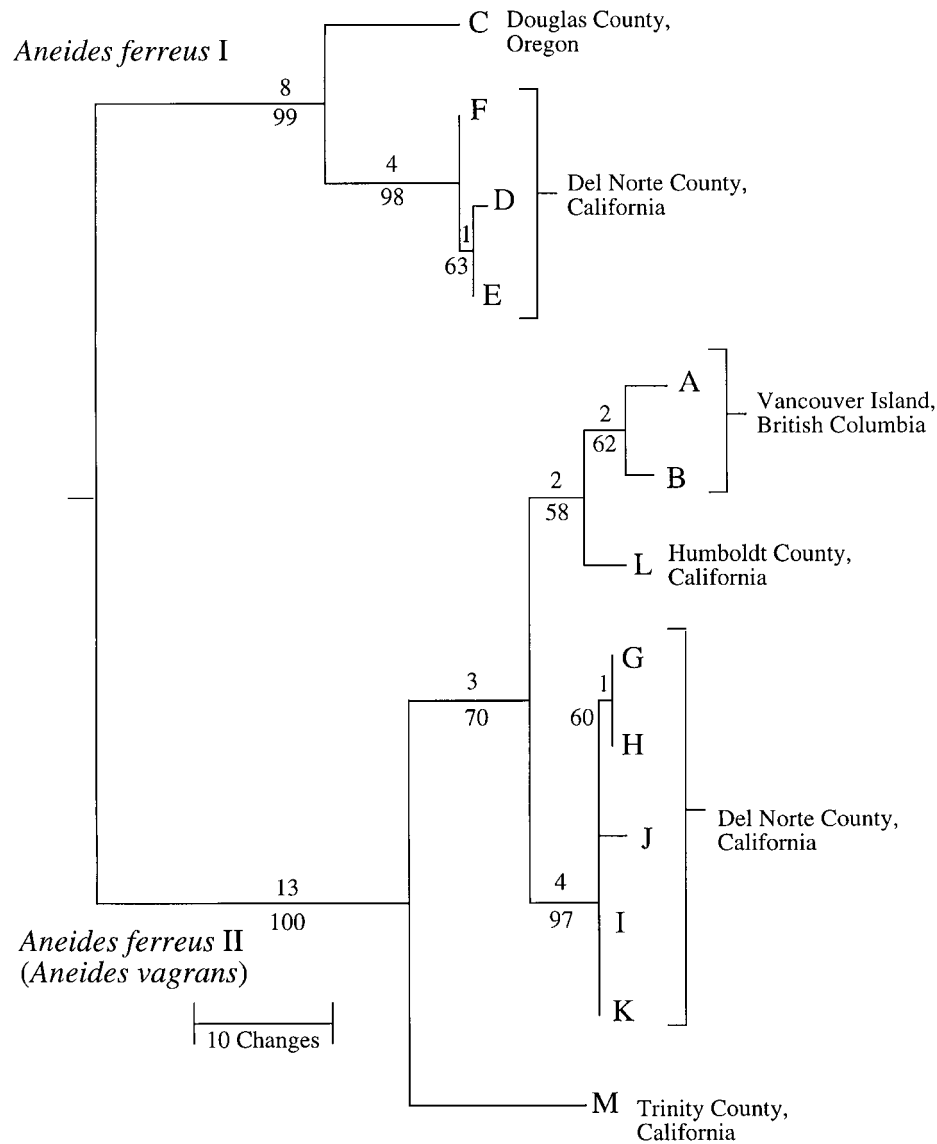
The results from ND2 are entirely consistent with the results of partial cytochrome *b* sequences (Jackman 1993). Cytochrome *b* showed little phylogenetic resolution within *ferreus* I and *ferreus* II, but showed an average of 9.6% divergence between the two, similar to the ND2 sequences reported here. The different sequences could not be combined

because the samples were from different individuals and showed differing amounts of missing data.

Historical results

Tan oak, or tan bark oak, can be found in the coastal forests of California, in southern Oregon, and in isolated patches in the north and central Sierra Nevada (Griffin and Critchfield 1972; Poulik et al. 1991). It is most abundant in the coastal forests of northern California, where it was once

Fig. 5. The single most parsimonious tree using all DNA data, unweighted. The decay index is indicated above each node and the bootstrap value below. The tree is 170 steps long. The branch lengths are proportional to the maximum number of possible changes. The outgroup (*Aneides flavipunctatus*) is not shown. Letters correspond to individuals from populations shown in Figs. 1 and 2.



one of the main lumber products. In 1878 there were 46 leather tanneries in the San Francisco bay area and 6 on Vancouver Island. There were also leather tanneries in Portland, Oregon, and Seattle, Washington (Hittell 1882). During this time, tan oak was cut extensively along the California coast. For example, from Shelter Cove, a relatively small port in Humboldt County, 2000 cords (256 000 cubic feet) of tan oak were shipped in 1878 (Hamm 1890). Other ports shipped out equally large, or larger, amounts of the bark annually (Bauer 1957). Nearly all of the bark logged north of San Francisco was shipped without being treated with harsh chemicals (Bauer 1957).

The stripping and transporting of bark was done in such a way that the inclusion of clouded salamanders seems probable (Jepson 1911; Fig. 6). In California, bark was stripped during the summer months, from May to September. This is a time when clouded salamanders are generally dormant in

California, but rains are not unusual, especially in May along the coast. The bark was stripped from fallen or standing trees. It was then cut into 4 by 2 ft strips, stacked and "...the bark is left in stacks for two to three weeks" before being bundled and transported to sea ports. The bark often sat for many months at the port before being shipped (West Coast Star 1883, in Bauer 1957).

In an 1880 letter, William Heathorn, one of the owners of a large tannery on Vancouver Island, wrote of his tannery, "...the bark used is chiefly hemlock, being supplemented by oak bark from California." He wrote that his tannery consumed over 500 tons of bark per year. Furthermore, he reported that two other tanneries only used oak bark from California. These statistics refer to the year 1878. If these tanneries used as much bark as the tannery owned by William Heathorn did, then at least 500 tons of oak bark were imported to Vancouver Island every year, as of 1878.

Fig. 6. Stacking of tan oak bark. Before being shipped, the bark was stacked and left for 2 weeks (Plate 2 in Jepson 1911).



The existence of two species of clouded salamanders

There are two well-differentiated groups within what is known today as *A. ferreus*. These two groups can be diagnosed by six fixed allozyme differences and 27 unambiguous codon changes for the last half of the mitochondrial ND2 gene, the tryptophan tRNA, and a portion of the cytochrome *b* gene. In addition, the two groups have well-differentiated chromosomes, with inversion, C-banding, and nucleolar organizer region (NOR) position differences (Sessions and Kezer 1987).

In the region of contact sampled, hybridization appears to be limited in scope despite the distribution of animals in apparently continuous habitat. The two groups have had a long separate history and species status is justified using either reproductive (i.e., the biological species concept; Mayr 1963) or phylogenetic (Cracraft 1983; Frost and Hillis 1990) criteria. The type locality of *A. ferreus* is Fort Umpqua, Oregon (Cope 1869). Therefore, *A. ferreus* II requires a new species name (Appendix 1).

It is possible that there are ecological differences between the two species of clouded salamanders. *Aneides ferreus* are commonly found on rocky slopes and under bark in Oregon, but are found almost exclusively under bark and in logs in California. This may be due to a difference in the abundance of rocky habitats in California and Oregon, or it may be that

A. ferreus in Oregon occupies places that *A. flavipunctatus*, the most terrestrial of the western *Aneides* species, normally occupies in California.

Introduction to Vancouver Island

A recent introduction to Vancouver Island from California poses some interesting and difficult questions, from both a historical and an ecological standpoint. First, *A. ferreus* has been reported from 23 of the small islands off the coast of Vancouver Island, and even on the mainland in one isolated spot. Some of these islands are small and uninhabited by people (Davis 1991). Also, clouded salamanders are abundant in low-elevation forests throughout Vancouver Island. The six tanneries in existence during the nineteenth century were located in Victoria, Westminster, and Nanaimo, all on the southeast coast of Vancouver Island. By 1906, the salamanders must have made it to at least one of the small islands, and in the last hundred or so years have spread and become abundant throughout Vancouver Island. The rapid spread of salamanders on Vancouver Island might be explained by the extensive logging activity there; salamanders might have been spread by moving cut trees. Their presence on the many small islands could be explained by rafting on debris left over from logging operations that was washed out to sea by storms. I have observed large log rafts in the ocean

with grass growing on the top side of the logs, indicating that they have not been saturated by seawater and would be suitable for salamanders.

In addition to the rapid spread of animals, there are differences in the life history and behaviors of Vancouver Island animals relative to California animals. In California, clouded salamanders are active at the surface during winter and spring, aestivating during the relatively dry summer months. On Vancouver Island, however, Davis (1991) has documented a reversal of this activity pattern, animals being dormant during the cold winter months and active at the surface primarily during the summer. Also, Vancouver Island animals lack the aggressive behavior seen in California animals. Behavioral studies by Davis (1991) show that *A. ferreus* is less aggressive than *Plethodon vehiculum* on Vancouver Island, and that biting events rarely occurred in *A. ferreus* Staub (1989), on the other hand, reported that one-third of all adult *A. ferreus* examined in California had head scars, presumably from conspecific bites. These differences imply a plasticity in behavior and activity patterns. The introduction to Vancouver Island can be considered to be a 100-year "greenhouse experiment" in which genetically similar groups have been growing in two different environments. The introduction allows the exploration of phenotypic plasticity in many traits on a large scale.

In the future, the following hypotheses need to be tested: (i) salamanders were introduced to Vancouver Island in a single event from the vicinity of Willow Creek (population 19); (ii) hybridization is restricted in all areas of contact between *A. ferreus* I and *A. ferreus* II; (iii) despite the overall similarity of *A. ferreus* I and *A. ferreus* II, there are consistent differences in morphology and ecology between them that have not yet been discovered.

Summary

There are two distinct historical entities within what is now known as *Aneides ferreus*. These two groups can be diagnosed by allozyme, mitochondrial DNA, and karyotypic characters, but not, as yet, by morphological characters. The two groups come into contact in northwestern California, where hybridization is limited. The California and Canadian populations are currently without a name, and one is provided in Appendix 1. Vancouver Island populations of salamanders were probably introduced from California with shipments of tan oak bark in the nineteenth century.

Acknowledgments

I thank David Wake for his support and help on this project. I thank Ted Davis for his help on Vancouver Island. I thank Allan Larson and Robert Macey for their help in the laboratory. I thank the Simpson–Rellim Lumber Company. I thank Megan Jackman, Elizabeth Jockush, Mario Garcia-Paris, Steve Deban, and Tyrone Hays for help in the field. This research was funded by National Science Foundation grants to David B. Wake.

References

Bauer, P.M. 1957. Bibliography of the history of tanning in California to 1900. Bancroft Library Archives, Berkeley, Calif.

- Beatty, J.J. 1983. Morphological variation in the clouded salamander, *Aneides ferreus* (Cope) (Amphibia: Caudata: Plethodontidae). Ph.D. dissertation, Oregon State University, Corvallis.
- Bremer, K. 1994. Branch support and tree stability. *Cladistics*, **10**: 295–304.
- Brown, W.M. 1985. The mitochondrial genome of animals. In *Molecular evolutionary genetics*. Edited by R.J. MacIntyre. Plenum Corp., New York. pp. 95–130
- Cope, E.D. 1869. A review of the species of the Plethodontidae and Desmognathidae. *Proc. Acad. Nat. Sci. Phila.* **21**: 93–118.
- Cracraft, J. 1983. Species concepts and speciation analysis. *Curr. Ornithol.* **1**: 159–187.
- Davis, T.M. 1991. Natural history and behaviour of the salamander, *Aneides ferreus* Cope. M.Sc. thesis, University of Victoria, Victoria, B.C.
- Felsenstein, J. 1993. PHYLIP (phylogeny inference package) version 3.5. Department of Genetics, University of Washington, Seattle.
- Fitch, W.M., and Margoliash, E. 1967. Construction of phylogenetic trees. *Science* (Washington, D.C.), **155**: 279–284.
- Frost, D. R., and Hillis, D. M. 1990. Species in concept and practice: herpetological applications. *Herpetologica*, **46**: 87–104.
- Griffin, J.R., and Critchfield, W.B. 1972. The distribution of forest trees in California. Pacific South West Forest and Range Experiment Station, Forest Service, U.S. Department of Agriculture, Berkeley, Calif.
- Hamm, L.E. 1890. History and business directory of Humboldt County. Daily Humboldt Standard Office, Eureka, Calif. p. 109.
- Harris, H., and Hopkinson, D.A. 1976. Handbook of enzyme electrophoresis in human genetics. North-Holland, Amsterdam.
- Highton, R. 1989. Biochemical evolution in the slimy salamanders of the *Plethodon glutinosus* complex in the eastern United States. I. Geographic protein variation. III. *Biol. Monogr.* No. 57.
- Highton, R., and Larson, A. 1979. The genetic relationships of the salamanders of the genus *Plethodon*. *Syst. Zool.* **28**: 579–599.
- Hittell, J.S. 1882. The commerce and industries of the Pacific coast of North America: comprising the rise, progress, products, present condition, and prospects of the useful arts on the western side of our continent, and some account of its resources: with elaborate treatment of manufactures, briefer consideration of commerce, transportation, agriculture, and mining: and mention of leading establishments and prominent men in various departments of business. A.L. Bancroft and Co., San Francisco.
- Jackman, T.R. 1993. Evolutionary and historical analyses within and among members of the salamander tribe Plethodontini (Amphibia: Plethodontidae). Ph.D. dissertation, University of California, Berkeley.
- Jackman, T.R., and Wake, D.B. 1994. Evolutionary and historical analysis of protein variation in the blotched forms of salamanders of the *Ensatina* complex (Amphibia: Plethodontidae). *Evolution*, **48**: 876–897.
- Jepson, W.L. 1911. California tanbark oak. U.S. For. Serv. Bull. No. 75.
- Kezer, J., and Sessions, S.K. 1979. Chromosome variation in the plethodontid salamander, *Aneides ferreus*. *Chromosoma*, **71**: 65–80.
- Larson, A. 1980. Paedomorphosis in relation to rates of morphological and molecular evolution in the salamander *Aneides flavipunctatus* (Amphibia: Plethodontidae). *Evolution*, **34**: 1–17.
- Lessa, E. 1990. Multidimensional analysis of geographic genetic structure. *Syst. Zool.* **39**: 242–252.
- Macey, J.R., Larson, A., Ananjeva, N.B., Fang, Z., and Papenfuss, T.J. 1997a. Two novel gene orders and the role of light-strand

- replication in rearrangement of the vertebrate mitochondrial genome. *Mol. Biol. Evol.* **14**: 91–104.
- Macey, J.R., Larson, A., Ananjeva, N.B., and Papenfuss, T.J. 1997b. Evolutionary shifts in three major structural features of the mitochondrial genome among iguanian lizards. *J. Mol. Evol.* **44**: 660–674.
- Maniatis, T., Fritsch, E.F., and Sambrook, J. 1982. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Press, New York.
- Moritz, C., Schneider, C.J., and Wake, D.B. 1992. Evolutionary relationships within the *Ensatina eschscholtzii* complex confirm the ring species interpretation. *Syst. Biol.* **41**: 273–291.
- Murphy, R.W., Sites, J.W., Buth, D.G., and Haufler, C.H. 1996. Proteins: isozyme electrophoresis. In *Molecular systematics*. Edited by D.M. Hillis, C. Moritz, and B.K. Maple. Sinauer Associates, Sunderland, Mass. pp. 51–69.
- Nei, M. 1972. Genetic distance estimates between populations. *Am. Nat.* **106**: 283–292.
- Nei, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics*, **89**: 583–590.
- Peabody, F.E., and Savage, J.M. 1958. Evolution of the coast range corridor in California and its effect on the origin and dispersal of living amphibians and reptiles. In *Zoogeography*. Edited by C.L. Hubbs. American Association for the Advancement of Science, Washington, D.C. pp. 159–186.
- Poulik, B.M., Muick, D.C., Johnson, S.G., and Popper, M. 1991. *Oaks of California*. Cachuma Press, Los Olivos, Calif.
- Saitou, N., and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**: 406–425.
- Selander, R.K., Smith, M.H., Yang, S.Y., Johnson, W.E., and Gentry, J.B. 1971. Biochemical polymorphism and systematics in the genus *Peromyscus*. I. Variation in the old-field mouse (*Peromyscus polionotus*). *Stud. Genet. (Univ. Tex. Publ.)* No. 6. pp. 49–90.
- Sessions, S.K., and Kezer, J. 1987. Cytogenetic evolution in the plethodontid salamander genus *Aneides*. *Chromosoma*, **95**: 17–30.
- Slevin, J.R. 1928. The amphibians of western North America. *Occas. Pap. Calif. Acad. Sci.* No. 16.
- Staub, N. 1989. The evolution of sexual dimorphism in the salamander genus *Aneides* (Amphibia: Plethodontidae). Ph.D. dissertation, University of California, Berkeley.
- Stebbins, R.C. 1951. *Amphibians of western North America*. University of California Press, Berkeley.
- Stebbins, R.C. 1985. *A field guide to western reptiles and amphibians*. Houghton Mifflin Co., Boston.
- Swofford, D.L., 1992. PAUP: phylogenetic analyses using parsimony, version 3.1. Computer Program Distribution. Illinois Natural History Survey, Champaign.
- Swofford, D.L., and Selander, R.B. 1981. A computer program for the analysis of allelic variation in genetics. *J. Hered.* **72**: 281–283.
- Wake, D.B., and Yanev, K.P. 1986. Geographic variation in allozymes in a “ring species,” the plethodontid salamander *Ensatina eschscholtzii* of western North America. *Evolution*, **40**: 702–715.

Appendix 1 by David Wake and Todd Jackman

Description of a new species of plethodontid salamander from California.

Aneides vagrans, new species Wandering salamander

HOLOTYPE: Museum of Vertebrate Zoology (MVZ) 124876, an adult male from a point about 10 km S Maple Creek, Humboldt Co., California, 40°42'N, 123°50'W, ca. 500 m elevation, collected on March 8, 1970, by J.F. Lynch, L.D. Houck, and M. Stevens.

PARATYPES: MVZ 124877–124883 (7 total), same data as holotype.

DIAGNOSIS: A moderately large (to about 80 mm snout–vent length) species of *Aneides* that is similar to *A. ferreus* in general morphology and osteology, but differs from that taxon in allozymes, mitochondrial DNA sequences (described in this paper), and karyological features (males of *A. vagrans* all have a telocentric chromosome 13, while females are either identical with males or are heteromorphic for an apparent inversion in chromosome 13 involving a metacentric state; *A. ferreus* is polymorphic for chromosome 13, which is either telocentric or subtelocentric; the species also differ with respect to the distribution of cytologically distinct NORs and C-band heterochromatin distribution; Sessions and Kezer 1987). The new species differs from co-occurring species of *Aneides* as follows: from *A. flavipunctatus* by its somewhat smaller size and its much longer limbs and digits, as well as differences in coloration (*A. vagrans* is mottled with shades of light to dark gray; *A. flavipunctatus* is shiny black, often overlain by lighter bronze or gray–green, and with spots of white to cream or yellow–white of varying size); from *A. lugubris* by its smaller adult size, less stocky habitus, somewhat longer trunk, and differences in coloration (*A. lugubris* is distinctly brown with discrete cream to yellow spots of varying size and density).

DESCRIPTION: This species has a stocky habitus with very long limbs and digits. The limbs usually touch and often overlap when adpressed to the side of the trunk (especially in individuals with a shorter trunk, those having 15 and 16 trunk vertebrae). The digits are long and cylindrical, but bear greatly expanded terminal pads that contain Y-shaped terminal phalanges. The first digit of both the hand and the foot is relatively much reduced in size, while the outermost digit is long and well developed. The head is very broad (somewhat broader in males than in females) and the jaw muscles are greatly enlarged. The maxillary and mandibular teeth are large, flattened, and few in number (on the order of 5 or 6 per side). Combined premaxillary and maxillary teeth average about 14.4 and 16, and combined anterior vomerine teeth average about 11 (from Beatty 1983). The tail is circular in cross section and while relatively short (always shorter than snout–vent length in adults), is strongly tapered, prehensile, and rarely broken. The ground color of adults is dark brown or gray with irregular mottling or marbling of lighter gray (illustrated by Stebbins 1951, Fig. 18.11). The mottling is especially prominent in populations from coastal Del Norte County, California.

COMPARISONS: Virtually all of the literature comparing *A. ferreus* from California and British Columbia with other species of salamanders refers to *A. vagrans*. The only spe-

cies, other than *A. ferreus*, with which *A. vagrans* is likely to be confused are *A. flavipunctatus*, which is typically larger, with an apparently longer trunk and shorter, stouter tail and much shorter limbs, as well as having a distinctly black, as opposed to dark brown, ground color, and *A. lugubris*, which is also larger and stouter but has a distinctly lighter brown ground color and some small but discrete yellowish to cream spots.

It is impossible, in our experience, to distinguish *A. ferreus* and *A. vagrans* on the basis of coloration or general appearance. However, we have the impression that *A. vagrans* has more striking coloration, especially in Del Norte County, where the two species come in contact. The marbling effect is pronounced in coastal and northern populations of *A. vagrans*, which gives them a contrasting black and gray pattern. The color pattern of *A. ferreus* in this region is a diffuse mix of brown and gold, which gives an impression of a clouded overlay on a dark ground color. These differences appear not to hold true outside the immediate area of contact, and local populations may have unique color patterns. A detailed morphometrical analysis of variation in *A. vagrans* and *A. ferreus* by Beatty (1983) disclosed substantial variation. Most of the 10 populations studied could be distinguished statistically from one another, but only 39% of individuals were correctly classified a posteriori. Only three populations were indistinguishable statistically, one from Vancouver Island and two from Humboldt County, California.

HABITAT: The new species is found almost exclusively under the bark of downed logs and under logs, but very rarely under rocks or on rocky slopes, a common habitat for *A. ferreus*.

RANGE: Almost all localities from which *A. ferreus* has been reported in California pertain to *A. vagrans*. The point

of contact between the two species is near the Smith River in northern California. The northernmost locality of *A. vagrans* known to us is on the south side of the Smith River within 3 km west of the junction with the South Fork of the Smith River. In contrast, *A. ferreus* extends south as far as the junctions of Hurdygurdy Creek and Goose Creek with the South Fork of the Smith River. The total zone of overlap is thus less than 15 km, but since *A. vagrans* lies to the south and west of the Smith River and its South Fork and *A. ferreus* lies to the north and east of this area, the two come together only along Rock Creek, which enters the South Fork of the Smith River from the west. There is evidence of some introgression between the two forms, but no clear hybrid individuals have been identified. Of 52 individuals found in the vicinity of Bald Ridge and Rock Creek, 10 (19%; 4 *A. ferreus* and 6 *A. vagrans*) showed some introgressed alleles, and all of these were males. There is a small zone of sympatry along Rock Creek. The two species also likely come into contact along the Klamath River between Happy Camp and Willow Creek, California, but details of distribution have yet to be determined.

Aneides vagrans extends from northern Siskiyou and Del Norte counties, California, south through extreme western Trinity, Humboldt, and Mendocino counties in an increasingly narrow coastal strip of well-forested habitat to the vicinity of Stewart's Point, northwestern Sonoma County, California. The species is widespread on Vancouver Island and neighboring islands in British Columbia, and has also been found on the mainland. We believe that all of these populations are derived ultimately from human-mediated introductions from California.

ETYMOLOGY: The name *vagrans* is derived from the Latin word *vagus*, "wandering," in reference to the colonizing ability of this species.