AN ABSTRACT OF THE THESIS OF

Douglas James DeGross for the degree of Master of Science in Environmental Sciences presented on April 16, 2004

Title: Gene Flow and the Relationship of *Plethodon stormi* and *P. elongatus* Assessed with 11 Novel Microstellite Loci

Redacted for privacy

Abstract approved

Stevan J. Arnold

Plethodontid salamanders are model organisms for studying evolutionary questions of gene flow, restricted ranges, and speciation. *Plethodon*, a diverse genus within Plethodontidae, is widely distributed in eastern and western North America. Much research has been based in the eastern United States where diversity of these salamanders is high. Because of their morphological conservatism, genetic analysis has often been used to address the questions of population structure, speciation, and gene flow. The majority of these analyses have relied on allozyme and mtDNA markers. However, these markers have their shortcomings in resolving differentiation on a small geographic scale. To date no researchers have used nuclear microsatellites to address systematic questions in plethodontid salamanders.

I developed 11 nuclear microsatellite loci to address evolutionary questions in two western members of the genus *Plethodon*, the sister species *Plethodon elongatus* and *P. stormi*. The taxonomic status of these salamanders has been a source of contention. Although recent mtDNA analyses reveal the presence of a third species previously included as *P. stormi* in the Scott River drainage, no samples from this possible new species are included in this thesis. Substructure inferred from the mtDNA analyses within the range of *P. stormi* is assessed, as well as gene flow between *P. elongatus* and *P. stormi*. My results provide strong support for treating *P. elongatus* and *P. stormi* as separate species.

Gene Flow and the Relationship of *Plethodon stormi* and *P. elongatus* Assessed with 11 Novel Microsatellite Loci

by

Douglas James DeGross

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Douglas James DeGross, Author

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Douglas J. DeGross, Louise S. Mead, and Stevan J. Arnold

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Abstract

Eleven tetranucleotide microsatellite loci were developed for the Del Norte salamander (*Plethodon elongatus*). The loci were variably polymorphic, ranging from two to 20 alleles per locus, with expected heterozygosities ranging from 0.07 to 0.86. The loci also amplified in a congener, the Siskiyou Mountain Salamander (*P. stormi*). The microsatellite loci will be used to assess the utility of highly polymorphic markers to assay within and between species differentiation between these two closely related species.

Introduction

Plethodontid salamanders are model organisms for addressing a variety of questions in evolutionary biology (Highton and Larson 1979, Wake and Larson 1987, Arnold *et al.* 1993, Houck and Verrell 1993). Of particular interest are contact zones that provide windows on evolutionary processes important in speciation (Harrison 1993, Highton 1995). Most studies of plethodontid contact zones have used either allozymes or mitochondrial genes. However, it would be advantageous to use more variable nuclear markers to analyze complex patterns of gene flow across species' boundaries (Wake and Jockusch 2000; Mead *et al.* 2001). Microsatellites can fill this role, but have never been used to analyze plethodontid contact zones. Here, we developed 11 microsatellite markers in order to clarify patterns of gene flow at the contact zone between two closely related western North American species of plethodontid salamanders (*Plethodon elongatus* and *P. stormi*).

Materials & Methods

Microsatellites were developed from two unrelated *P. elongatus* individuals (MEP0133 and LSM0267). Genomic DNA was extracted using a proteinase K digestion and phenol-chloroform technique (Hillis *et al.* 1996) from tail tissue removed non-lethally from each salamander in the field. We used a degenerate oligonucleotide primer polymerase chain reaction [DOP-PCR] to develop the microsatellite markers following the general procedure of Cabe and Marshall (2001). This method employs a degenerated primer (K6-MW), and a gradient of annealing temperatures to amplify many genomic fragments from 200-2000 bp (Macas *et al.* 1996). The DOP-PCR conditions were identical to those used by Hoffman *et al.* (2003). PCR fragments were enriched for microsatellite repeats by hybridization to biotynilated $GATA_8$ oligonucleotides, using the conditions of Hoffman *et al.* (2003). These repeat rich fragments were captured using streptavidin-coated magnasphere beads (Promega Corporation, Madison, WI). A second enrichment step was carried out following the procedure of Cabe and Marshall (2001). The eluate from this second enrichment was run in a final DOP-PCR to create double stranded product which was cloned and plated using a Topo cloning kit (Invitrogen Corporation, Carlsbad, CA), and subsequently incubated overnight at 37°C. Colonies were screened using the PCR protocol of Cabe and Marshall (2001). Of the 480 colonies screened, approximately 145 positive colonies were isolated (30% efficiency) and were purified using a PCR

Purification kit (QIAGEN Inc., Valencia, CA). Purified samples were quantified and sent to the Nevada Genomics Center (University of Nevada, Reno, NV) and sequenced in one direction with the sequencing primer T7.

Thirty-seven unique sequences had microsatellite motifs and adequate flanking regions for primer design. Eleven of the designed primer pairs amplified successfully and so the forward primer was fluorescently labeled to test for locus variability. Samples of both *P. elongatus* and the congeneric species *P. stormi* successfully amplified using the following PCR conditions: 94°C for 5 mins, 35X (94°C for 1.5 mins, locus specific annealing temperature (Table 1.1) for 45 s, and 72°C for 45 s); and 72°C for 10 mins. Amplified DNA was analyzed for fragment size in the ABI 3100 capillary system at Oregon State University.

Table 1.1: Microsatellite primers from the Del Norte Salamander (*Plethodon elongatus*) with results of loci analyses for *P. elongatus* and it's congeneric species *P. stormi*

Locus	Sequence(5'-3')	Repeat Motif	Annealing Temp. (C)	GenBank Accession #	*P. elongatus* Ho	*P. elongatus* He	*P. elongatus* Size Range	*P. stormi* Ho	*P. stormi* He	*P. stormi* Size Range
***Plel* 100**	*F-ATGAAATGGCACGACTTGTTTA									
	CACATGGGGTAGGAAATGACTT	$(TCTA)_{23}$	53	AY532595	0.733	0.802	296-344	0.800	0.859	276-302
***Plel* 101**	*H-TATGCGAGTTATCTCACTATGC									
	TTGTACTGTCCATTGCTTTCT	$(AGAT)_{12}$	56	AY532596	0.800	0.763	160-180	0.600	0.660	164-200
***Plel* 103**	*F-AGCACAGTCCCAACCTCAAATC									
	CCATGCGAATGTGGCACTATAC	$(TCTA)_{30}$	56	AY532597	0.667	0.761	181-215	0.300	0.741	173-201
***Plel* 104**	*F-GATGCTGCTTTGGAAATGTGT									
	CGCCTCGAAATTACTTATAGTTGT	$(GATA)_{15}$	62.5	AY532598	0.467	0.521	261-281	0.700	0.794	261-315
***Plel* 105**	*H-CCTGCTGTCAAGTACCAT									
	CTACGCTCTATTCAAAATCA	$(ATCT)_{19}$	61	AY532599	0.867	0.747	284-334	0.600	0.782	292-314
***Plel* 107**	*F-CTGGGGATTTATCGCTAGTC									
	CAGGCAGGGGTAAAAACTAT	$(AGAT)_{16}$	61	AY532600	0.600	0.568	427-459	0.400	0.400	403-407
***Plel* 108**	*F-GGGGTGGCAGCTGTAGACT									
	ATACGTCATGCCGCCCAGTAGTTA	$(GATA)_{8}$	62	AY532601	0.200	0.186	210-214	0.400	0.471	210-214
***Plel* 109**	*H-GAGCCGATCCAAGCGAGGT									
	AGGGGGAGCACGGACTTGT	$(GATA)_{5}$	61.5	AY532602	0.067	0.067	265-269	0.700	0.729	261-301
***Plel* 110**	*H-CTATTTGACTGAACCTGTA									
	CAAGTCCCTCTATGTAGTA	$(GATA)_{18}$	55	AY532603	0.667	0.691	326-368	0.800	0.841	318-368
***Plel* 111**	*F-GTCTCACCCCACTCACTTTGCTA									
	GTATGTCCACTGCTCGTCTTTCTT	$(TAGA)_{22}$	55	AY532604	0.800	0.853	342-368	0.500	0.406	334-342
***Plel* 112**	*F-GGCTGTGGAATATGTACTTT									
	GTGGAGTGGCGAGAGAGA	$(TCTA)_{14}$	59	AY532605	0.467	0.531	365-385	0.700	0.782	369-417

*** 5' flourescently labeled primers (*F=6-FAM & *H=5-HEX (Qiagen 100 Atlantic Ave. Alameda, Ca. 94501))**
Ho = Observed Heterozigosity
He = Expected Heterozigosity
Size Range = Base pair size of representative alleles

Results and Discussion

All loci were analyzed in Genepop vers. 3.3 (Raymond and Rousset 1995). One population of *P. elongatus* (n=15) and one population of *P. stormi* (n=10) were used to estimate allelic frequencies and to test for Hardy-Weinburg (HWE) and genotypic equilibria (exact probabilities). Sequential Bonferroni corrections for multiple comparisons were applied to both equilibrium tests (Sokal and Rohlf 1995). The 11 scored loci are polymorphic with 2 to 20 alleles per locus (mean alleles/locus = 9.7) in the combined sample of *P. elongatus* and *P. stormi* (Table 1.1). Expected heterozygosities (H_E) range from 0.067 to 0.853 (mean H_E = 0.590) for *P.elongatus*, and from 0.400 to 0.859 (mean H_E = .679) for *P.stormi* (Table 1.1). Neither of the populations was out of HWE (P>0.05), and there was no evidence for linkage disequilibrium (P>0.05). These loci represent the first microsatellite markers to be diagnosed for any of the western North American plethodontids.

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Gene Flow and the Relationship of the Salamanders *Plethodon stormi* and *P. elongatus* Assessed with 11 Novel Microsatellite Loci

Douglas J. DeGross

Abstract

Plethodon salamanders are model organisms for the study of gene flow and speciation. Two western members of this genus are a case in point. The systematic relationship of *Plethodon elongatus* and *P. stormi* has been controversial for decades. With the advent of highly variable nuclear microsatellite markers, these questions can now be addressed. Use of 11 microsatellite markers demonstrates limited gene flow between *P. elongatus* and *P. stormi* in a contact zone in western Siskiyou County, California. These results agree with the evolutionary picture drawn from previous allozyme and mtDNA analyses. These species have apparently been geographically separated and have recently come back into contact in the vicinity of Happy Camp and Seiad Valley, California. The results support mtDNA haplotype groupings of *P. elongatus* and *P. stormi* populations into sister species. Population clusters within each species are recognizable from both mtDNA and microsatellite analyses. Furthermore, both analyses support designation of two lineages within *P. stormi* that form a monophyletic group in relation to *P. elongatus*. There is support from these results to designate the *P. stormi* clades as Evolutionary Significant Units (ESU's), but further sampling is needed to define their contact zone and to clarify the amount of gene flow present between the mtDNA clades of *P. stormi*.

Introduction

The systematics of plethodontid salamanders are of general interest because major evolutionary questions can be addressed in this diverse family (Wake 1993). Salamanders of this family are models for evolutionary studies because of their morphological conservatism, prevalence of cryptic species, and presence of species with restricted ranges (Wake and Yanev 1986, Wake *et al* 1989, Hairston 1992, Arnold *et al* 1993, Wake 1993, Arnold 2002). The genus *Plethodon*, in particular, has provided case studies for understanding reproductive isolation, contact zones and speciation (Hairston 1949, 1992, Highton 1995, Highton and Peabody 2002).

The systematic status of the salamanders *Plethodon elongatus* and *P. stormi* has been controversial for decades. Described by Van Denburgh (1916), *P. elongatus* is widespread along the coast of southern Oregon and northern California, throughout the Siskiyou-Klamath region. Brodie (1971b) recognized inland populations as *P. stormi*. These populations differed from *P. elongatus* in coloration, vertebral number and limb length (Brodie 1970, Brodie and Storm 1971a). Currently known localities for the two species are as little as 0.4 km apart, and in some places contact is separated by fifth order streams within the same watershed (Thompson Creek, Jackson County; Oregon and Indian Creek, Siskiyou County, California). Highton and Larson's (1979) allozyme survey indicated that

P.elongatus and *P. stormi* were sister species. This observation, coupled with an apparent overlap in morphological characters across a contact zone in western Siskiyou County (personal experience and communication with field surveyers), California, suggested that the two forms might be intergrading subspecies.

In 1994 impetus for focus on the relationship between *P. elongatus* and *P. stormi* was created by the implementation of the federal Northwest Forest Plan (NWFP) (USDA and USDI 1994). The NWFP mandated field sampling and site management of over 400 rare and little known species associated with old growth forest ecosystems on federal lands within the range of the Northern Spotted Owl (*Strix occidentalis*). The creation of a "Survey and Manage" list of species also identified rare taxa warranting study due to insufficient population and ecological data for effective management. Both *P. elongatus* and *P. stormi* were placed on this list. As a consequence, extensive sampling for these two species was conducted throughout their ranges in the Klamath-Siskiyou region beginning in 1995.

With increased sampling, it became apparent that the ranges of *P. elongatus* and *P. stormi* met at multiple locations and that intergradation might occur. In western Jackson County, OR, where the two species came into close proximity, they were physically very different, and identification was straightforward. However, in western Siskiyou County, CA, field identification was problematic. At this and other sites, field

identification suggested that the two forms might hybridize or that two reproductively isolated forms might have converged in morphology. Genetic analysis was initiated to assess population structure and to designate species boundaries. Surveys conducted for NWFP implementation facilitated collection of tissues for genetic analyses across the range of both species in Oregon and California.

Molecular studies focused on mtDNA haplotypes and inferred population substructure within both *P. elongatus* (Mahoney 2004) and *P. stormi* (Pfrender and Titus 2001, Mead *et al.* in review). The most comprehensive analysis of *P. stormi* supports the recognition of three distinct mtDNA clades (Mead *et al.* in review). Both Mahoney (2004) and Mead *et al.* (in review) discuss a basal lineage within the complex. This lineage, with a geographic range of approximately 200 km^2, is currently being proposed as a new species (Mead *et al.* in review). No samples from that clade were included in this analysis. The other two clades are included in an effort to understand their relationships, and will be referred to as *P. stormi* north (N) and *P. stormi* south (S) (Figure 2.1). *P. stormi* N populations are found in the Applegate Valley south to upper Seiad Valley and the Siskiyou crest, occupying an area of approximately 300 km^2. *P. stormi* S populations are found along the northern banks of the Klamath River west to the *stormi-elongatus* range boundaries along Indian Creek in western

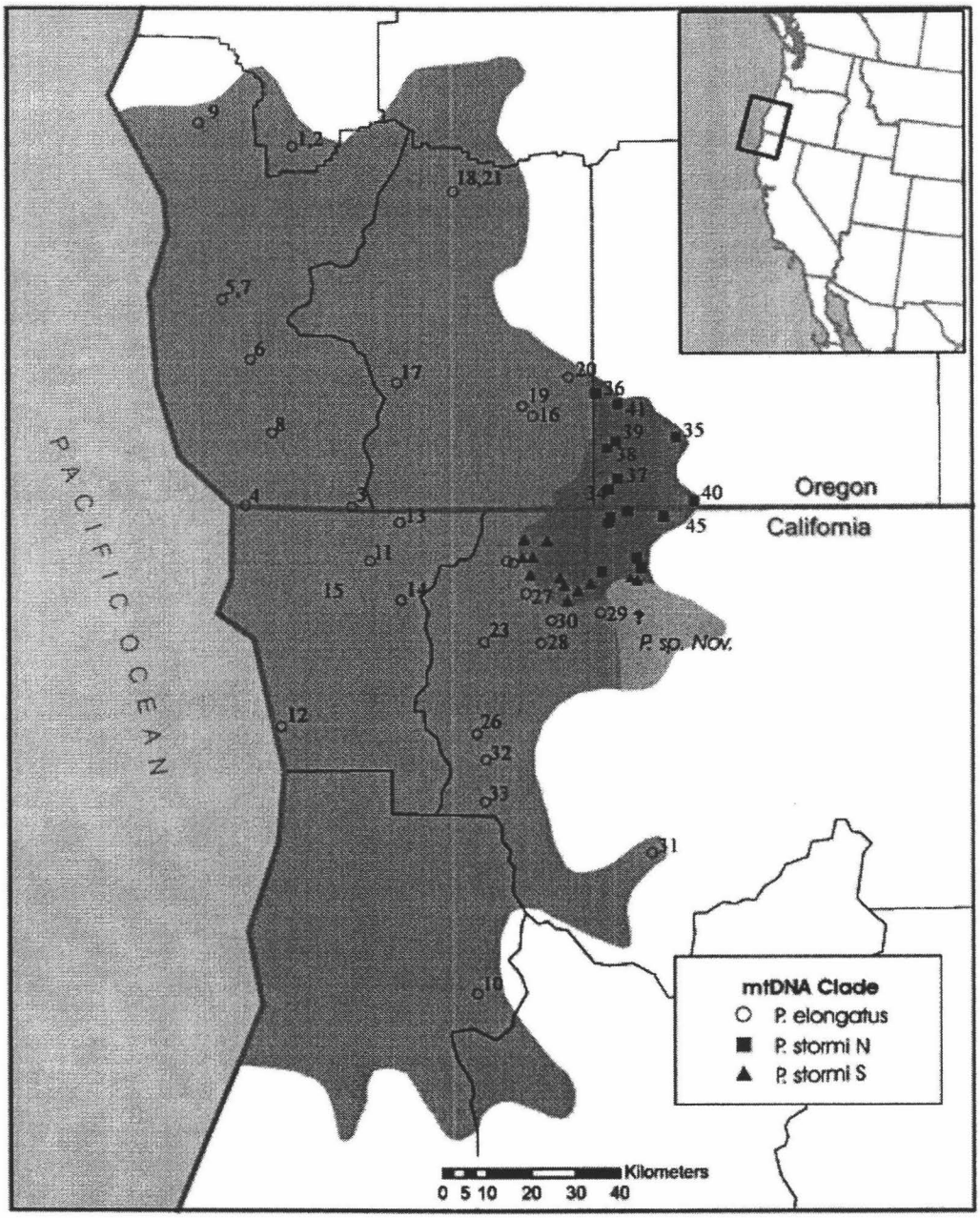

Figure 2.1: Regional map denoting the current species ranges for *P. elongatus* (medium grey), *P. stormi* (darker grey), and a proposed new species not included in this analysis (light grey). The three mtDNA haplotype clades in this analysis are represented by open circles (*P. elongatus*), solid squares (*P.stormi* N), and solid triangles (*P. stormi* S). Each locality is designated by its associated reference number (Table 2.1).

Siskiyou County, occupying a range of approximately 100 km^2 (Figure 2.1). In contrast, the range of *P. elongatus* encompasses an area of roughly 19,000 km^2. Do the two mtDNA haplotype clades of *P. stormi* represent subspecies of *P. elongatus*, located on the inland periphery of its range, or are they a reproductively isolated species with small geographic ranges?

Although allozyme and mtDNA studies have identified *P. elongatus* and *P. stormi* as sister taxa and elucidated their population structure, the issue of gene flow between the two forms has not been addressed. The southwestern contact zone is of particular interest because of the morphological similarities of *P. elongatus* and *P. stormi* in that region (personal experience and personal communication with field crews). Does hybridization occur in the contact zone between *P. elongatus* and the *P. stormi* S mtDNA clade? The present analysis was conducted to address the question of gene flow at this contact zone and to evaluate the relationships of populations of *P. stormi* S with the other mtDNA clades.

Materials & Methods

Specimens and DNA extraction

Tissue samples were collected from 203 individual *Plethodon elongatus* (100) and *P. stormi* (103) at 60 localities in Coos, Curry, Jackson, and Josephine Counties in Oregon, and Del

Table 2.1: Specimen list with reference numbers for maps, locality, locality code, species, and mtDNA clade designations based on Mahoney (2004), Mead *et al* (in review), and unpublished data.

	Locality	Code	Species	mtDNA clade	County	State	Latitude N	Longitude W	Mahoney samples	Mead et al samples	Sample Size
1	Coquille	COQ	Plel	E	Coos	OR					1
2	Daphne Grove	PRS	Plel	E	Coos	OR	42.735	124.053			5
3	North Fork Diamond Creek	DIA	Plel	E	Curry	OR	42.000	123.880			1
4	Mosier Creek	MOS	Plel	E	Curry	OR	42.000	124.170			1
5	Prehistoric Amphitheatre	PHA	Plel	E	Curry	OR	42.420	124.240			2
6	East Fork Pistol River	PIS	Plel	E	Curry	OR	42.300	124.160			1
7	Quosatana Butte	QSB	Plel	E	Curry	OR	42.420	124.240			2
8	Snake Tooth Butte	STB	Plel	E	Curry	OR	42.150	124.100			1
9	Sixes River	SXR	Plel	E	Curry	OR	42.780	124.310			1
10	Hoopa	HOP	Plel	E	Del Norte	CA	41.007	123.535			1
11	Patrick Creek	PAC	Plel	E	Del Norte	CA	41.890	123.830	x		1
12	Requa	REQ	Plel	E	Del Norte	CA	41.549	124.067	x		1
13	Stateline Rest Area	SRA	Plel	E	Del Norte	CA	41.970	123.750	x		12
14	South Fork Smith River	SSR	Plel	E	Del Norte	CA	41.811	123.745			1
15	3 mi SW of Gasquet	GAS	Plel	E	Del Norte	CA	41.598	123.541	x		1
16	Bear wallow	BWA	Plel	E	Josephine	OR	42.190	123.390			1
17	Near Fiddler Gulch	FID	Plel	E	Josephine	OR	42.255	123.760		x	1
18	Graves Creek	GVC	Plel	E	Josephine	OR	42.651	123.583		x	1
19	Paradise Mine	PAR	Plel	E	Josephine	OR	42.210	123.416			1
20	Powell Creek	POW	Plel	E	Josephine	OR	42.270	123.290		x	8
21	Rainie Falls	RAF	Plel	E	Josephine	OR	42.647	123.608		x	1
22	Bald Hornet Creek	BHC	Plel	E	Siskiyou	CA	41.418	123.432			3
23	Clear Creek	CLC	Plel	E	Siskiyou	CA	41.727	123.519		x	5
24	Deadman East	DEE	Plel	E	Siskiyou	CA	41.888	123.439			1
25	Deadman West	DEW	Plel	E	Siskiyou	CA	41.892	123.457			3
26	Dobbins Creek	DOC	Plel	E	Siskiyou	CA	41.539	123.538		x	15
27	Doolittle Creek	DOO	Plel	E	Siskiyou	CA	41.826	123.406			3
28	Elk Creek/Twin Creek	ELC	Plel	E	Siskiyou	CA	41.726	123.365	x	x	15
29	Grider Creek	KLA	Plel	E	Siskiyou	CA	41.787	123.205		x	1
30	Ottley Gulch	OTG	Plel	E	Siskiyou	CA	41.771	123.336		x	6
31	Sawyers Bar	SAW	Plel	E	Siskiyou	CA	41.297	123.064	x	x	1
32	Sandy Bar Creek	SBC	Plel	E	Siskiyou	CA	41.487	123.513	x	x	1
33	Somes Bar (2 mi NW)	SMB	Plel	E	Siskiyou	CA	41.402	123.515		x	1
34	Carberry Creek	CBC	Plst	N	Jackson	OR	42.040	123.181	x	x	1
35	China Gulch	CHG	Plst	N	Jackson	OR	42.062	123.157		x	1
36	Ferris Gulch	FRG	Plst	N	Jackson	OR	42.238	123.218	x	x	1
37	Grouse Creek	GRC	Plst	N	Jackson	OR	42.148	122.998	x	x	1
38	Little Humpy	LHM	Plst	N	Jackson	OR	42.134	123.225	x		1
39	NineMile Creek	NMC	Plst	N	Jackson	OR	42.139	123.163	x	x	4
40	Yellow Jacket	YJK	Plst	N	Jackson	OR	42.017	122.948	x	x	1
41	Hinkel Gulch	HKG	Plst	N	Jackson	OR	42.227	123.163	x		1
42	Applegate Bridge	ABR	Plst	N	Siskiyou	CA	41.984	123.176		x	6
43	Baker Gulch	BG	Plst	S	Siskiyou	CA	41.865	123.395			9
44	East Fork Indian Creek	EFI	Plst	S	Siskiyou	CA	41.9	123.415	x	x	1
45	Elliot Creek	ELI	Plst	N	Siskiyou	CA	41.986	123.031	x	x	6
46	Evans Mountain	EVM	Plst	S	Siskiyou	CA	41.833	123.265		x	6
47	Horse Creek Northern	HCR	Plst	S	Siskiyou	CA	41.9	123.104		x	5
48	Horse Creek Southern	HCR	Plst	S	Siskiyou	CA	41.881	123.090		x	3
49	Horse Camp Trail	HCT	Plst	N	Siskiyou	CA	41.972	123.183		x	1
50	Joe Miles	JMC	Plst	S	Siskiyou	CA	41.813	123.294		x	7
51	Joe Creek	JOE	Plst	N	Siskiyou	CA	41.995	123.130	x	x	9
52	Kuntz Creek	KZC	Plst	?	Siskiyou	CA	41.789	123.091			2
53	Lower Devil's Peak	LDP	Plst	N	Siskiyou	CA	41.875	123.202			5
54	Rhodonite Creek	RHO	Plst	S	Siskiyou	CA	41.901	123.388			10
55	Schutts Gulch	SCG	Plst	S	Siskiyou	CA	41.831	123.136			2
56	Thompson Creek (1)	SFI	Plst	S	Siskiyou	CA	41.937	123.411			1
57	Slide Creek	SLC	Plst	S	Siskiyou	CA	41.869	123.324			4
58	Seattle Creek	STC	Plst	S	Siskiyou	CA	41.843	123.301	x	x	9
59	Thompson Creek (2)	TC	Plst	S	Siskiyou	CA	41.935	123.349		x	1
60	West Grider	WGR	Plst	S	Siskiyou	CA	41.848	123.231		x	5

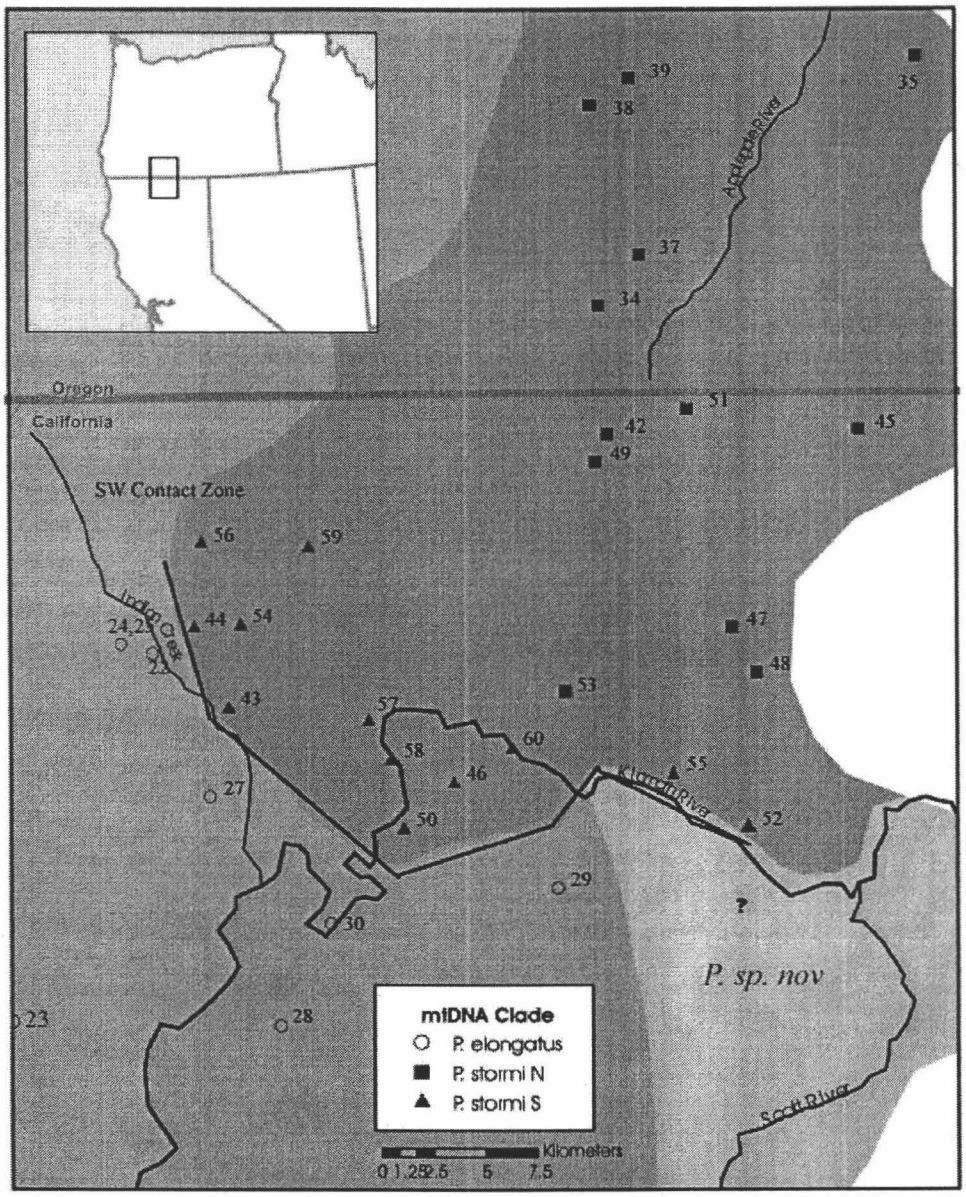

Figure 2.2: Fine scale map of contact zone between *P. stormi* and *P. elongatus*. MtDNA haplotype clades are represented by open circles (*P. elongatus*), solid squares (*P.stormi* N (I)), and solid triangles (*P. stormi* S (II)). Localities denoted by reference numbers (Table 2.1).

Norte and Siskiyou Counties in California (Table 2.1 & Figs. 2.1 & 2.2). One hundred and three *P. stormi* specimens from 27 localities were used to represent the geographic range of the species: 46 individuals from *P. stormi* N and 57 individuals from the *P. stormi* S. One hundred *P. elongatus* specimens from 33 localities were sampled from the northern two-thirds of the species range, and most are representative of Mahoney's *P. elongatus* group 1 mtDNA clade. Fifty-five *P. elongatus* specimens came from localities in close proximity of the southwest zone of contact with *P. stormi* S (Figure 2.2). This contact zone can be found in the immediate vicinity of the Happy Camp, California and the confluence of Indian Creek with the Klamath River (Figure 2.2). Three *P. stormi* S populations (46, 50, and 60) are located south of the Klamath River, between Happy Camp and Seiad Valley, California within a northern encroaching meander of the river. The western boundary of this contact zone lies along Indian Creek, which is exclusive to Siskiyou County, California. Specimens of the *P. stormi* S clade are currently believed to reside on the eastern side of Indian Creek, with *P. elongatus* located to the west. The results from Mead *et al* (2004) support this geographic barrier, but sampling was not extensive within the drainage.

Genomic DNA from all specimens was extracted from tail tissue with a standardized protocol (Hillis *et al.* 1996). A 3-5mm piece of tail tissue was taken from individuals in the field. Tail samples were placed in 70% ethyl alcohol and stored at -80°C. Genomic DNA was extracted from tissue samples using a proteinase K digestion and phenol-chloroform technique (Hillis et al. 1996). Eppendorf's PhaseLock Gel tubes (Eppendorf AG, Barkhausenweg 1, 22339 Hamburg Germany) were used during DNA extraction, and therefore only a single phenol-chloroform wash was necessary. The resulting genomic DNA from each specimen was used as template for the subsequent PCR.

Microsatellite Development and Genotyping

Microsatellites were developed from two unrelated *P. elongatus* individuals (MEP 0133 and LSM 0267) (DeGross *et al* 2004). We used a degenerate oligonucleotide primer polymerase chain reaction [DOP-PCR] to develop the microsatellite markers following the general procedure of Cabe and Marshall (2001). PCR fragments were enriched for microsatellite repeats by hybridization to biotynilated $GATA_8$ oligonucleotides, using the PCR conditions of Hoffman et al. (2003). The eluate was cloned and plated using a Topo cloning kit (Invitrogen Corporation, Carlsbad, CA). Colonies were screened using the PCR protocol of

Cabe and Marshall (2001). Positive microsatellite colonies were isolated with approximately 30% efficiency. Purified specimens were quantified and sent to the Nevada Genomics Center (University of Nevada, Reno, NV) and sequenced in one direction with the sequencing primer T7.

Thirty-seven unique sequences had microsatellite motifs and flanking regions adequate for primer design. Eleven of the designed primer pairs amplified successfully. The forward primer was fluorescently labeled to test for locus variability. Specimens of both *P. elongatus* and the congeneric species *P. stormi* successfully amplified (DeGross *et al* 2004). Amplified DNA was analyzed for fragment size in the ABI 3100 capillary system at Oregon State University's Central Services Lab. The genotypes of each individual were then determined using the program GENOTYPER 2.5.2 (ABI Prism).

Analysis of Genetic Variation

All loci were analyzed in Genepop vers. 3.1d (Raymond and Rousset 1995) to assess Hardy-Weinberg equilibrium (HWE) and linkage equilibrium. One population of *P. elongatus* (n=15) and one population of *P. stormi* (n=10) were used to estimate allelic frequencies and to test for (HWE) and linkage equilibrium with exact probabilities. Sequential Bonferroni corrections for

multiple comparisons were applied to both equilibrium tests (Sokal and Rohlf 1995). Neither of the populations was out of HWE (P>0.05), and there was no evidence for linkage disequilibrium (P>0.05) (DeGross *et al.* 2004). Therefore the assumption was made that these markers could be used to assay neutral variation across the two species, and to infer relatedness and gene flow. The allelic diversity within loci, heterozygosities, pairwise estimates of F_{st} (Weir and Cockerham 1984) and R_{st} (Slatkin 1995) were calculated in Genepop vers. 3.3.

Bayesian Assignment and Genetic Admixture Analyses

STRUCTURE (Pritchard *et al.* 2000) was used to infer population structure, conduct multilocus assignment tests, and to infer gene flow by identification of specimens with admixed genomes. This program uses Bayesian methods to probabilistically assign specimens to populations and assess the amount of population admixture using allelic identities at multiple loci. We used 100,000 iterations with a 10,000 iteration burn-in for all analyses. In preliminary trials, an increase to one million iterations and a 100,000 iteration burn-in period did not substantially change estimated likelihoods.

To assess population admixture, we ran additional analyses with prior population designations. Prior population designations

were based on mtDNA haplotype analyses (Mahoney 2004, and Mead *et al.* in review), as well as unpublished mtDNA haplotype data for the samples from this analysis. Using those designations, simulations were run to infer two, three, and four populations to reflect the presence of two species, as well as three and four mtDNA clades. For the Bayesian assignment analysis, we removed the prior population designations and ran identical simulations, allowing the program to compute the proportion of each individual's genome that could be assigned to each of *K* hypothetical populations. In addition, parallel analyses were run with just the two *P. stormi* clades to assess their level of differentiation.

Canonical Discriminant Analysis

A canonical discriminant analysis was conducted using CANDISC in SAS 8e (1999 SAS institute, Inc. Cary N.C.) to assess the degree of microsatellite differentiation among mtDNA clades. This procedure derives linear combinations (canonical variables) of original quantitative variables that maximize between-clade variation (CANDISC procedure). The analysis was conducted using the sum of the two allele sizes at each locus in each individual as quantitative variables. Because of missing

values, only 111 individual specimens with complete data for all 11 loci were available for this analysis (45 *P. elongatus*, 29 *P. stormi* N, and 37 *P. stormi* S).

Results

Analysis of Genetic Variation

The genotypes of 203 individuals were evaluated at 11 microsatellite loci. Although the loci were developed in *P. elongatus,* they amplified all specimens from the two *P. stormi* mtDNA clades. All of the microsatellites were polymorphic with 7 to 37 alleles per locus (mean = 20.82) (Table 2.2).

Many private alleles, unique within a group, were found in both *P. elongatus* (n = 61) and *P. stormi* (n = 45), with the number of private alleles per locus ranging from 0 to 17 (Table 2.2). Two loci in general had many private alleles. Seventeen of 37 alleles in Plel100 were found exclusively in *P. stormi*, with 6 private alleles found in each of the *P. stormi* clades. Sixteen of 31 Plel103 alleles were found exclusively in *P. elongatus* and 6 private alleles were found in each of the two *P. stormi* clades (Table 2.2). The

Table 2.2: Microsatellite locus specific variation in size (base pairs), total alleles and private alleles/locus for *P. elongatus, P. stormi,* and *P. stormi* mtDNA clades

Microsatellite Loci	Size range (bp)	Total alleles	Number of private alleles					
			P. elongatus (Total)		*P. stormi* (Total)		*P. stormi* N	*P. stormi* S
Plel100	244-350	37	5		17		6	6
Plel101	152-234	17	3		6		1	2
Plel103	157-243	31	16		7		0	0
Plel104	253-363	20	5		2		0	1
Plel105	256-354	21	2		2		0	2
Plel107	399-467	18	8		2		1	1
Plel108	206-230	7	0		2		1	0
Plel109	257-335	17	5		5		1	2
Plel110	304-400	24	8		1		1	0
Plel111	322-432	17	3		0		0	0
Plel112	349-433	20	6		1		0	0

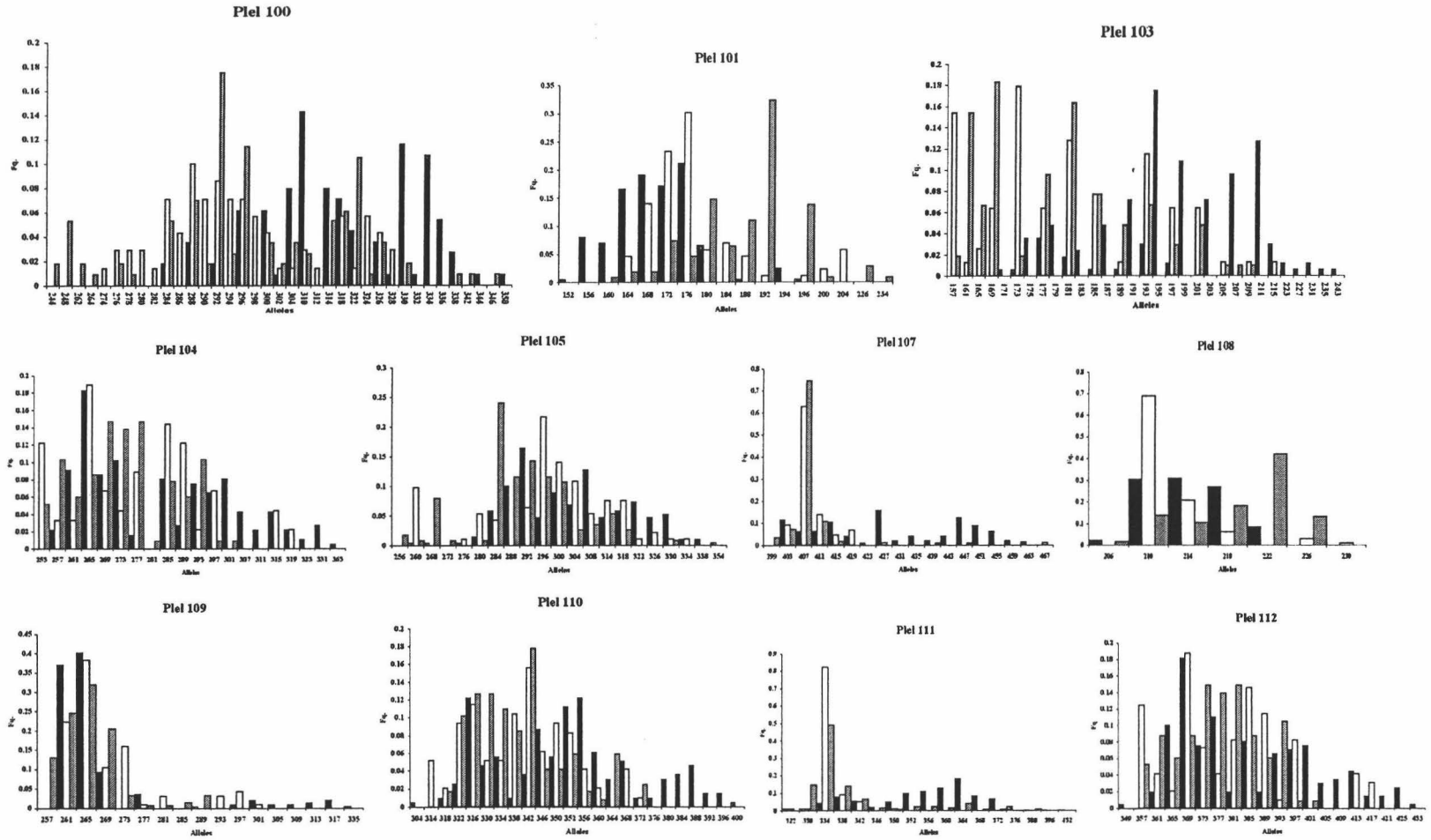

Figure 2.3: Eleven polymorphic microsatellites and their allelic frequency distributions in *P. elongatus* (black), *P. stormi* N (white), and *P. stormi* (grey).

remaining loci had many more private alleles within *P. elongatus* compared to the two *P. stormi* clades. Although there were fixed differences among the loci, there was also extensive overlap of allelic distributions.

Distributions of allele sizes showed marked differences between *P. elongatus* and *P. stormi* and between the two *P. stormi* mtDNA clades (Figure 2.3). *Plethodon elongatus* and *P. stormi* showed size differentiation at three loci (Plel100, Plel101, Ple103), whereas the two *P. stormi* clades were strongly differentiated at two loci (Plel101, Plel108). Compared with *P. elongatus*, both *P. stormi* clades showed leptokurtic distribution of allele sizes at two loci (Plel107, Plel111).

Expected heterozygosities in the three mtDNA clades ranged from 0.342 to 0.917 (Table 2.3) and were consistently greater than the observed heterozygosities. A deficit of heterozygotes occurred in every clade at all loci, and in some cases the deficiency was pronounced. This occurence is most likely do to our sampling and the presence of many populations within each mtDNA clade, a situation that will reduce the number of observed heterozygotes (Hartl and Clark 1997). Plel107 and Plel108 displayed striking heterozygote deficiencies across all clades, with observed values half of the amount expected.

Table 2.3: Expected (H_e) and observed (H_o) heterozygosities for 11 nuclear microsatellite loci in *P. elongatus*, *P. stormi*, and two *P. stormi* mtDNA haplotype clades.

	P. elongatus		*P. stormi* (all)		*P. stormi* N		*P. stormi* S	
Loci	**H_o**	**H_e**	**H_o**	**H_e**	**H_o**	**H_e**	**H_o**	**H_e**
Plel100	0.580	0.711	0.718	0.911	0.717	0.87	0.672	0.868
Plel101	0.480	0.817	0.544	0.809	0.587	0.737	0.475	0.725
Plel103	0.470	0.753	0.573	0.807	0.435	0.765	0.639	0.762
Plel104	0.610	0.851	0.641	0.901	0.565	0.855	0.656	0.849
Plel105	0.630	0.861	0.689	0.897	0.674	0.879	0.656	0.812
Plel107	0.520	0.867	0.184	0.473	0.217	0.441	0.148	0.467
Plel108	0.280	0.717	0.252	0.766	0.283	0.486	0.213	0.724
Plel109	0.270	0.672	0.456	0.802	0.435	0.782	0.447	0.746
Plel110	0.720	0.916	0.748	0.947	0.761	0.915	0.688	0.908
Plel111	0.440	0.857	0.407	0.577	0.304	0.342	0.459	0.679
Plel112	0.650	0.906	0.67	0.927	0.587	0.893	0.688	0.862
Mean	***0.514***	***0.812***	***0.535***	***0.802***	***0.506***	***0.724***	***0.522***	***0.764***

Table 2.4: F_{st} and R_{st} for all pairwise mtDNA clade comparisons between *P. elongatus, P. stormi* N, and *P. stormi* S.

	Statistic	*P. elongatus*	*P. stormi S*
P. elongatus	F_{st}	-	0.0772
***P. stormi* (all)**		0.0702	-
P. stormi N		0.0883	0.0586
P. stormi S		0.0772	-
P. elongatus	R_{st}	-	0.3154
***P. stormi* (all)**		0.3081	-
P. stormi N		0.2748	0.0597
P. stormi S		0.3154	-

Estimates of F_{st} (Table 2.4) showed the highest level of differentiation between *P. elongatus* and *P. stormi N* (0.088), a slightly lower level between *P. elongatus* and *P. stormi S* (0.077) and the lowest differentiation between the two *P. stormi* clades (0.059). The corresponding estimates of R_{st} were 0.315, 0.275 and 0.060 (Table 2.4).

Bayesian Assignment and Genetic Admixture Analyses

To compare the population structure based on the microsatellites with that based on mtDNA, we first computed population assignments with no prior mtDNA clade designations. Although the present analysis is restricted to microsatellite data, the inferred structure of mtDNA clades was used as a baseline for comparison. In assessment of the best fitting population model for these data, we varied *K* (the number of inferred populations) over the range 1-20. Those runs produced an enigmatic peak in likelihood at K=12. Thus, this analysis provided no internal basis for recognizing a particular number of populations.

In separate runs, we specified that STRUCTURE infer 1-4 hypothetical populations, based upon the presence of four mtDNA haplotype clades in the data set, and scrutinized the composition of those populations (Table 2.5). Specifying that two populations be inferred resulted in assignment of all *P. elongatus* to one

Table 2.5: Bayesian Assignment Analysis for 2, 3, and 4 inferred population clusters on full dataset (203 individuals at 11 nuclear loci) with no prior mtDNA clade designation, using the program STRUCTURE (Pritchard et al 2000). Values presented are *q* (probability of ancestry) for each mtDNA haplotype clade. The first three rows of values represent assignments when two clusters are inferred, the second set of three rows represents assignments when three clusters are inferred, and the final three rows represent assignments when four clusters are inferred. Boldface values represent the cluster associations with the highest probablities for each of the mtDNA haplotype clades.

	Inferred clusters			
mtDNA clade	**I**	**II**	**III**	**IV**
P. stormi N	0.099	**0.901**		
P. stormi S	0.112	**0.888**		
P. elongatus	**0.953**	0.047		
P. stormi N	0.056	**0.860**	0.084	
P. stormi S	0.072	**0.838**	0.090	
P. elongatus	**0.476**	0.030	**0.494**	
P. stormi N	0.032	**0.693**	0.043	**0.231**
P. stormi S	0.062	**0.325**	0.08	**0.532**
P. elongatus	**0.477**	0.047	**0.447**	0.03

population and nearly all *P. stormi* (regardless of mtDNA clade, N or S, identity) to the other. Specifying that three populations be inferred resulted in roughly equal assignment of *P. elongatus* into two populations, and all *P. stormi* (regardless of clade identity) to a third population. Specifying that four populations be inferred resulted in roughly equal assignment of *P. elongatus* to two inferred populations. The other two inferred populations to which *P. stormi* mtDNA clades (N and S) were assigned showed variable levels of identity for each clade. The mean of *P. stormi* N specimens showed 0.693 probability of assignment into one of the populations, and *P. stormi* S specimens produced a probability of 0.532 into the other population (Table 2.5). This analysis suggests that differentiation within *P. elongatus* is more profound than the differentiation between *P. stormi* N and *P. stormi* S.

Restricting the assignment analysis exclusively to the *P. stormi* samples produced a best-fit model of nine populations (maximum likelihood at $K = 9$). The samples group into eight geographically contiguous populations plus one inferred population that encompasses unassociated samples from three different corners of the species range. This analysis demonstrates the fine scale geographic resolution possible with these microsatellites within *P. stormi*.

Estimates of genome admixture also were made in the course of the assignment analysis with no prior designations of mtDNA clade identity. Using the estimates under the specification that four populations be inferred, 5% of genomes in *P. elongatus* represent admixture with *P. stormi* N and 3% represent admixture with *P. stormi* S genomes (Table 2.5). About a quarter of *P. stormi* N represent admixture with *P. stormi* S and 8% represent admixture with *P. elongatus*. About a third of *P. stormi* S represent admixture with *P. stormi* N and 14% represent admixture with *P. elongatus*.

Analysis with prior mtDNA clade designations gave essentially the same results for admixture but also helped infer the extent of gene flow. A total of 21 specimens demonstrate varying levels of genomic admixture from the two inferred population analysis, in order to assess admixture at the species level (Table 2.6). Genomic admixture in these individuals may arise from gene flow or it may represent convergence in allele size. Individuals from localities at or near a contact zone with substantial evidence of genomic admixture (n=16), probably represent hybridization. In contrast, individuals (n=5) with substantial admixture from localities far from contact zones, probably represent convergence.

Table 2.6: Individuals from data set with admixed genomes as deduced from *q* (the probability of ancestry into inferred group) in Bayesian Admixture Analysis using the program STRUCTURE (Pritchard *et al* 2000). Individual sample numbers are given in parentheses in the first column. All individuals present in this table had *q* values < 0.85 for their respective mtDNA species designations.

Admixed genome	*q (P. elongatus)*	*q (P. stormi)*
	***P. stormi* N**	
ABR(162)	0.531	0.469
HRC(079)	0.596	0.404
HRC(080)	0.606	0.394
ABR(163)	0.723	0.277
	***P. stormi* S**	
WGR(007)	0.260	0.740
SCG(304)	0.293	0.707
SCG(305)	0.297	0.703
JMC(092)	0.298	0.702
SFI(091)	0.298	0.702
KZC(321)	0.467	0.533
WGR(011)	0.491	0.509
WGR(008)	0.836	0.164
BG(311)	0.970	0.030
BG(310)	0.981	0.019
	P. elongatus	
GAS(207)	0.795	0.205
QSB(165)	0.761	0.239
SAW(205)	0.727	0.273
OTG(128)	0.686	0.314
PHA(103)	0.641	0.359
MOS(212)	0.638	0.362
DOC(149)	0.613	0.387

Table 2.7: Canonical discriminant analysis of the three mtDNA clades using 11 microsatellite loci as variables

Variable	Raw Canonical Coefficients		Univariate Test Statistics	
	CAN1	**CAN2**	**F value**	**Pr > F**
Plel100	0.0057	0.0148	7.65	0.0008
Plel101	-0.0371	0.0214	62.80	<.0001
Plel103	0.0094	-0.0015	27.12	<.0001
Plel104	0.0287	0.0140	9.23	0.0002
Plel105	-0.0004	-0.0111	11.48	<.0001
Plel107	0.0147	0.0104	37.38	<.0001
Plel108	0.0029	0.0725	18.96	<.0001
Plel109	0.0086	-0.0208	4.38	0.0149
Plel110	0.0047	0.0092	8.65	0.0003
Plel111	0.0392	0.0070	46.29	<.0001
Plel112	-0.0148	0.0057	7.08	0.0013
(Canonical Correlation)^2	**0.7960**	**0.4164**		
Multivariate Statistics and F approximations				
	Value	**F value**	**Num/Den DF**	
Wilks' Lambda	0.1190	16.91	22/196	<.001
Pillai's Trace	1.2124	13.86	22/198	<.001

Canonical Discriminant Analysis

All eleven microsatellite loci provided significant univariate contributions to discrimination among the three mtDNA clades, with F ratios ranging from 4.38 to 62.80 ($p = 0.015$ to <0.0001). The R^2 values reported by the same procedure ranged from 0.075 for Plel109 to 0.538 for Plel101. Multivariate test statistics revealed significant differences between the mtDNA clades in microsatellite vectors. Four multivariate statistics (Wilks' Lambda, Pillia's Trace, Hotelling-Lawley Trace, and Roy's Greatest Root) were significant at the < 0.0001 level, but we report only the the F values of the most conservative statistics (Wilks' Lambda and Pillia's Trace) (Table 2.7). The first canonical variable CAN 1 (Table 2.7) accounted for the greatest differentiation among clades. The R^2 (Canonical Correlation^2) between the clades and CAN1 was 0.796, which is almost twice as large as the R^2 for CAN2, 0.416.

The clustering of the specimens in canonical space depicts the differentiation among mtDNA clades at the microsatellite loci (Figure 2.4). Individual outliers from each mtDNA clade are also indicated with arrows and locality numbers (Figure 2.4). These specimens, other than the Carberry Creek (34) specimen from

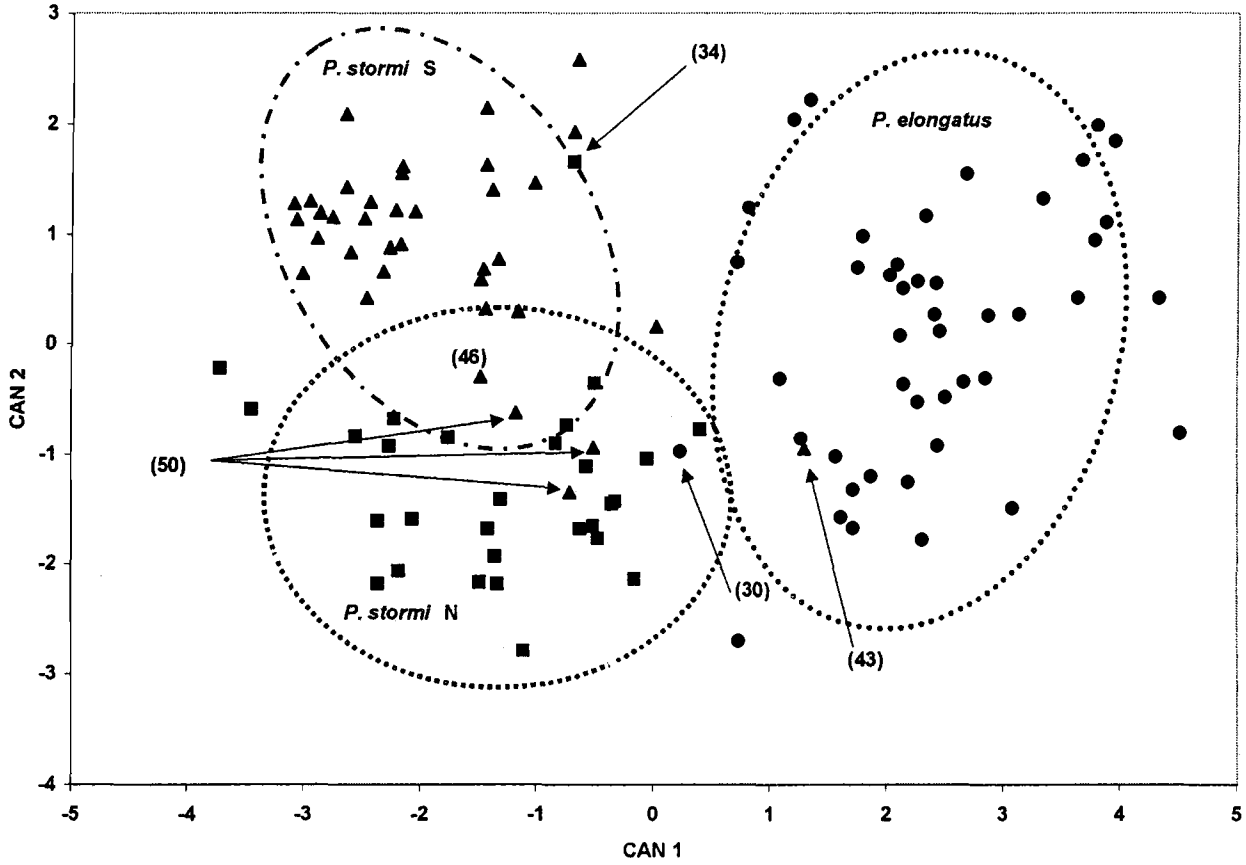

Figure 2.4: CDA ordination plot of three mtDNA clades (*P. elongatus* (circles), *P. stormi* N (squares), and *P. stormi* S (triangles)), with outlier specimens (locality code labels) indicated with arrows, 80% confidence ellipses are indicated.

P. stormi N, all originated within 3 km of the Klamath River and associated contact zones. The Baker Gulch (43) *P. stormi* S specimen that clustered with *P. elongatus* is one of the same specimens that received the inverse species assignment in the STRUCTURE analysis. Three of the *P. stormi* S specimens from Joe Miles (50) clustered near the center of the *P. stormi* N cluster and are the most geographically distant from the contact zone between the two *P. stormi* clades. The most noticeable outlier from the *P. elongatus* cluster is from Ottley Gulch (30) which lies approximately 8 km west of Joe Miles (50) along the Klamath River, and hence within the contact zone between *P.elongatus* and *P. stormi* S.

Discussion

The status of *Plethodon elongatus* and *P. stormi*

The use of microsatellites for analysis of relatedness and gene flow in this study are the first of their kind in plethodontid systematics. The ability of these highly variable nuclear markers to address issues of differentiation and gene flow on a small geographic scale is especially useful. Although the status of *P. elongatus* and *P. stormi* as sister taxa has been established (Highton and Larson 1979, Mahoney 2004, Mead *et al.* in review), questions persist about gene flow, in particular along the southwestern species contact zone in western Siskiyou County,

California. Morphological similarity along the Klamath River, in and around Happy Camp, California, has helped to cloud the relationship of these species (personal experience and personal communication with researchers). The use of 11 microsatellite loci to address these questions helps resolve the taxonomic controversy surrounding these species.

The F_{st} values (Table 2.4) for comparisons of *P. elongatus* and the *P. stormi* clades are within the standard range for moderate genetic differentiation (Hartl & Clark 1997). R_{st} values are much higher, indicating there is greater differentiation among mtDNA haplotype clades in allelic identity or size than in allele frequency (Slatkin 1995). Furthermore, the presence of 61 private alleles (species specific) in *P. elongatus* and 45 in the pooled *P. stormi* sample indicates that there is limited gene flow between *P. elongatus* and *P. stormi* (Randi *et al* 2001). Although the *P. elongatus* samples used in this analysis are almost exclusively drawn from the group 1 mtDNA haplotype clade of Mahoney (2004), population structure within *P. elongatus* was evident. Even though the substructure within the *P. elongatus* samples and the differences between the *P. stormi* clades are quite substantial, the data indicate that *P. elongatus* and *P. stormi* are separate species. Multivariate analyses of the 11 microsatellite loci lend

strong support to the view that *P. elongatus* and *P. stormi* are distinct species.

Limited gene flow occurs between *P. elongatus* and *P. stormi* in isolated populations in western Siskiyou County, California. The geography of these species suggests that they have come into secondary contact along their southwest species boundary in this region (Mahoney 2004). The limited number of individuals with admixed genomes at several contact zone populations (Figure 2.5) bolsters this view. These admixed genomes apparently do not represent samples from a hybrid swarm or zone of intergradation. In those cases we would expect to find uniformly high incidences of admixture in the contact zone. At localities (60 & 55), admixed genomes represent a minority of the sample. The samples that do not show admixed genomes demonstrate q values of > 0.95 for their respective species. The clearest evidence for genomic admixture, and hence for hybridization, occurs in specimens from drainages that flow into the Klamath River in the vicinity of the Seiad Valley, California. Here the three clades come into their closest proximity. The variable levels of genomic admixture within in these contact zone populations suggest limited hybridization rather than introgression.

The results from the admixture analysis demonstrated some interesting instances of mixed ancestry. Two specimens from a *P.*

stormi S population (43) in the Indian Creek drainage just north of Happy Camp, California, consistently clustered with very high probabilities (>0.985) within the *P. elongatus* cluster. The presence of two specimens with *P. elongatus* microsatellite profiles at Baker Gulch (43) may represent sympatry between *P. elongatus* and *P. stormi,* but this claim needs to be supported by further mtDNA analysis. Aside from these two specimens, no other individuals clustered convincingly with the wrong mtDNA clade. Some *P. elongatus* specimens geographically isolated from the *P. stormi* clades demonstrated low probabilities of mixed identity. These three specimens from near Gasquet (28), Quosatana Butte (7), and the Prehistoric Amphitheatre (5) are all from more coastal *P. elongatus* populations. The most likely explanations for these individuals are that they either have conserved ancestral alleles at a few loci, or convergently evolved allelic identities with *P. stormi.*

The status of *P. stormi* N and *P. stormi* S

Unlike the F_{st} and R_{st} values reported for the *P. stormi* clade comparisons with *P. elongatus*, the values recorded for the comparisons between *P. stormi* N and *P. stormi* S were consistently at the lower range of moderate differentiation (Table 2.4). The Bayesian Assignment Analysis suggests that the three mtDNA clades represent at least two distinct species. For

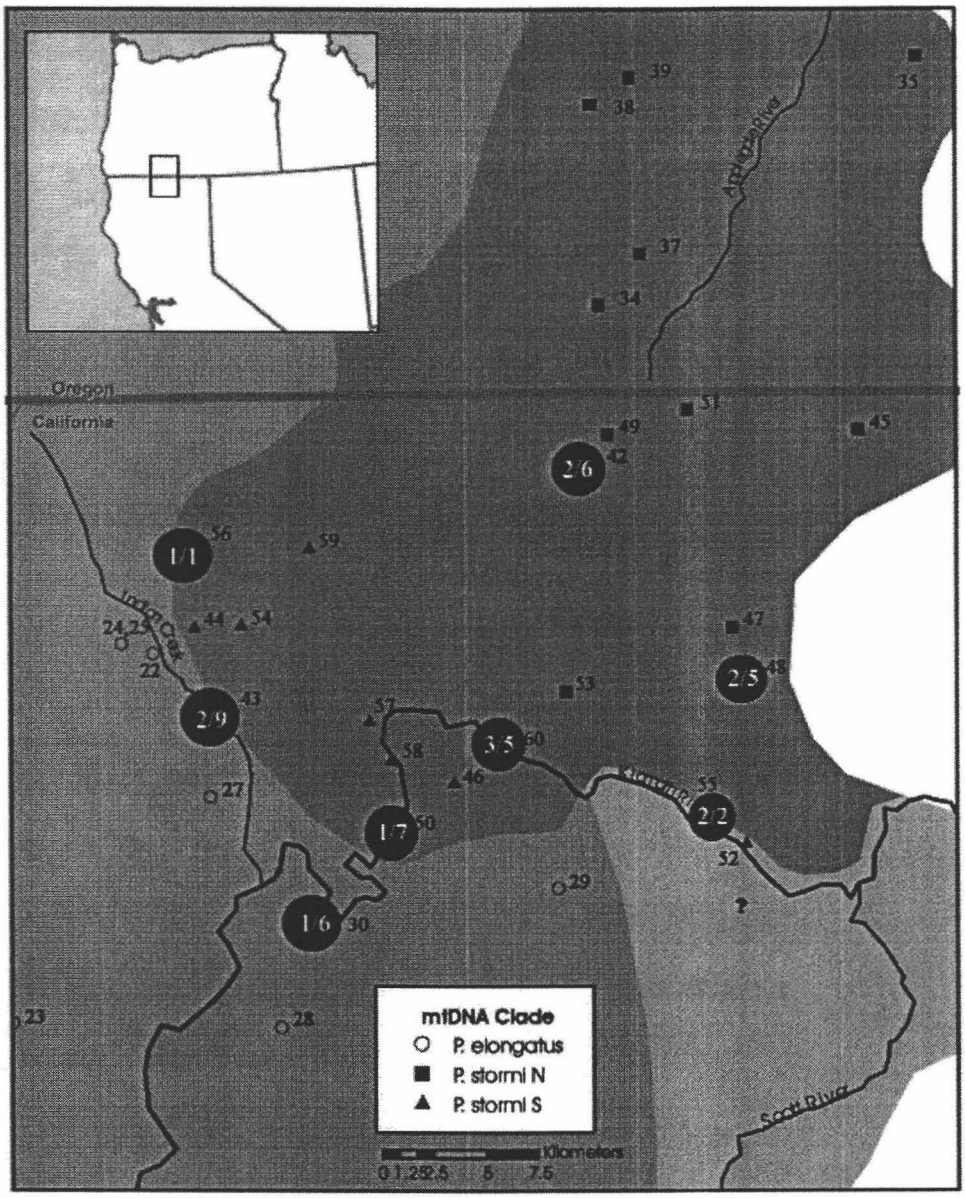

Figure 2.5 The southwestern contact zone in Siskiyou County, California, showing the fraction of admixed genomes at each site (30, 42, 43, 48, 50, 52, 56, and 60). Populations with samples having admixed genomes are denoted by large black circles. Values within circles are the number of admixed individuals over the total number sampled per locality. Remaining localities that are *P. elongatus* populations signified by open circles, *P. stormi* N signified by black squares, and *P. stormi* S by black triangles.

example, the hypothesis that the *P. stormi* S clade is a hybrid population between *P. stormi* N and *P. elongatus* is not supported by these analyses. Instead, the results indicate that the two *P. stormi* clades are more closely related to one another than to *P. elongatus*, in agreement with mtDNA analyses (Mahoney 2004, and Mead *et al.* in review). However, the microsatellite analysis also reveals population structuring within the *P. stormi* clades. Populations in close proximity cluster together. The nine inferred groupings of populations that best fit the *P. stormi* samples are geographically contiguous except for the ninth cluster. This ninth cluster incorporates geographically unassociated samples that did not fit into the other clusters. Furthermore, this ninth cluster includes several of the samples that demonstrate striking levels of admixture. The canonical discriminant analysis revealed strong separation of the two *P. stormi* clades on the second canonical variable. This multivariate analysis lends support to the separation of the two *P. stormi* clades as distinct, differentiated lineages, as well as additional support for *P. elongatus* and *P. stormi* as separate species.

The data presented here, support the presence of two differentiated groups within *P. stormi*, which further support the mtDNA analysis from Mead *et al* (2004). Although these two

groups may not be biological species these two units should receive recognition because of their ecological and evolutionary significance. Distinct differentiation of these two clades in their mtDNA and microsatellite loci warrants the designation of Evolutionary Significant Units (ESUs) (Moritz 1994). However, additional sampling and genetic analysis is needed to determine the zone of contact between *P. stormi* N and *P. stormi* S and to assess gene flow between these two clades.

Evolutionary Considerations

The Klamath-Siskiyou region is known for its geologic complexity and high levels of endemism (Coleman and Kruckeberg 1999). The region is tectonically active, and large-scale geologic events have changed the landscape in the last ten million years (Orr & Orr 2000). A marked gradient in moisture and daily temperature extremes is present from the moist coast east to the dry interior where *P. stormi* resides. The moister coast allows *P.elongatus* to be active on the surface for at least five months per year. In contrast, the more xeric conditions of the interior limit *P. stormi* surface activity to less than a month per year. The presence of many high elevation (>1,800 m) ranges and extensive river systems undoubtedly contribute to disjunction of terrestrial salamander populations and reproductive isolation.

Local differences in temperature and moisture, coupled with the geologic history of the region, are probably the driving forces that produced the marked population structure and interruption of gene flow that we observed in the *P. elongatus-stormi* complex. The existence of limited gene flow in this region, as well as the presence of a new form worthy of species status to the southeast confounds the current management regime for these salamanders (Mead *et al.* in review).

Our analyses support the prospect that plethodontid lineages with small geographic ranges can be reproductively isolated from other lineages. Although *P. stormi* S occupies a range of only about 100 km^2 the results from this analysis indicate that it is well differentiated from *P. elongatus* and the two clades engage in only limited hybridization. Intensive geographic sampling and genetic analysis in the plethodontid genera *Plethodon* and *Batrachoceps* have revealed several other instances of clades with restricted ranges ($\leq$ 200 km^2) that have attained species status. Examples include *Batrachoseps campi, Plethodon amplus, P. aureolus, P. cheoah, P. hubrichti, P. meridianus, P. sequoyah,* and *P. sp. nov.* (Marlow *et al* 1979, Highton *et al* 1989, Highton and Peabody 2000, Highton 2004). These reports and the

present results suggest that numerous geographically restricted species of plethodontids await discovery.

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