Molecular Ecology (2004) **13**, 149–166 doi: 10.1046/j.1365-294X.2003.02030.x

Molecular systematics and phylogeography of the *Plethodon elongatus* species group: combining phylogenetic and population genetic methods to investigate species history

MEREDITH J. MAHONEY
Museum of Vertebrate Zoology, 3101 VLSB, University of California, Berkeley, CA 94720–3160 USA

Abstract

***Plethodon elongatus* and *P. stormi* (Caudata: Plethodontidae) are Pacific Northwest endemic species which occur in northwestern California and southwestern Oregon. Studies on these salamanders have resulted in differing taxonomic conclusions, but the underlying historical hypotheses, at both inter- and intraspecific levels, have never been examined in a molecular framework. Here, representatives of 81 populations from throughout the range of both taxa are sequenced. Portions of three mitochondrial protein-coding genes (cytochrome *b*, NADH dehydrogenase subunit 4, and ATPase 6) were sequenced. Four haplotype groups with nonoverlapping geographical ranges were recovered in separate and combined analyses of the data. One clade corresponds to the distribution of *P. stormi*, while the remaining three comprise *P. elongatus*. Phylogenetic relationships among haplotype groups differ in separate analyses of the genes but converge on a well-supported topology, with *P. elongatus* and *P. stormi* as monophyletic sister taxa, in combined Maximum Parsimony and Maximum Likelihood analyses. Population genetic analyses of mismatch distributions and Tajima's *D*-statistic are consistent with range expansion for the largest clade within *P. elongatus*, covering the northern two-thirds of the species range. In contrast, the *P. stormi* haplotype clade and the *P. elongatus* clade from the southern third of the species range may have been relatively stable. Morphological boundaries between *P. elongatus* and *P. stormi* are largely congruent with mitochondrial DNA breaks and continued treatment as sister taxa is supported. Although mitochondrial DNA haplotype groups may reflect historical separation within *P. elongatus*, genetic barriers are incongruent with intraspecific patterns of morphological variation.**

Keywords: mtDNA, Pacific Northwest, phylogeography, *Plethodon*, population history, salamanders

Received 8 July 2003; revision received 3 October 2003; accepted 3 October 2003

Introduction

Hypotheses of biogeographic and evolutionary history are used regularly in molecular studies to generate testable predictions of genetic structure at shallow and deep levels of divergence (Riddle 1996; Avise 2000). While vicariance events and climatic cycles may be linked to deeper phylogenetic structure (Moritz *et al*. 1992; Avise 2000; Brunsfeld *et al*. 2001), demographic events such as growth or expansion following a bottleneck can leave signatures in both phylogenetic structure and genetic variation (Tajima 1989a; Slatkin & Hudson 1991; Rogers & Harpending 1992). Recent studies of mitochondrial DNA (mtDNA) variation combining phylogenetic and population genetic approaches have provided insight into patterns and processes at multiple levels in species history for a number of taxa from western North America (e.g. Conroy & Cook 2000; Nielson *et al*. 2001; Matocq 2002).

The salamanders *Plethodon elongatus* and *P. stormi* (Plethodontidae) are the only endemic amphibian taxa in the species-rich Klamath-Siskiyou region of southern Oregon and Northern California (Bury & Pearl 1999; DellaSala *et al*. 1999). *Plethodon stormi* is restricted to the Siskiyou Mountains, while *P. elongatus* has a larger range in southeastern

Correspondence: M. J. Mahoney. Fax: (510) 643—8238; E-mail: mmahone2@socrates.berkeley.edu

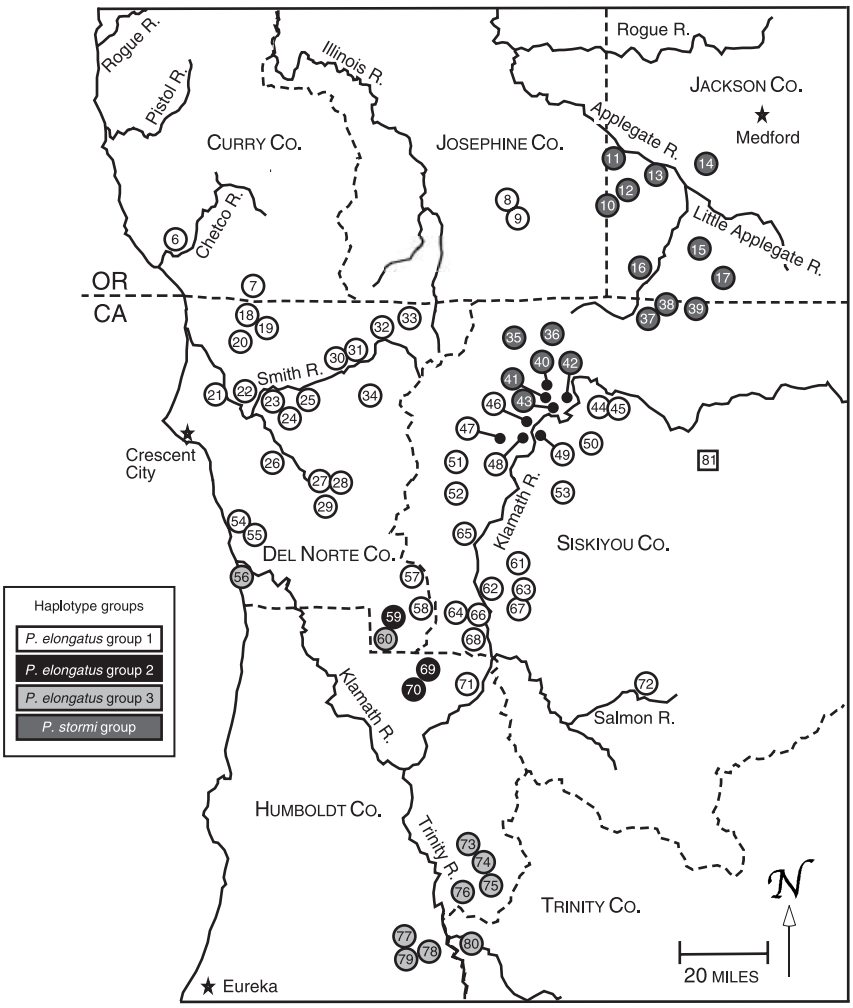

Fig. 1 Map of northwestern California and southwestern Oregon showing localities of samples sequenced for this study and haplotype group assignments for populations based on phylogenetic analyses. Dashed lines are county and state borders. Solid lines are major rivers. Population numbers as in Table 1. Population 1 (Coos County, OR), Populations 2 and 3 (Josephine County, OR), and Populations 4 and 5 (Curry County, OR), are north of the mapped area and are included in *P. elongatus* haplotype group 1. Population 81 (square) is phylogenetically distinct and is not assigned to one of the main haplotype groups.

Oregon and northwestern California (Brodie & Storm 1971; Brodie 1971; Stebbins 2003; Fig. 1). These closely related species (Highton & Larson 1979; Mahoney 2001) are distinguished by coloration, body proportions and number of vertebrae (Highton & Brame 1965; Brodie 1970; Nussbaum *et al*. 1983). There is local variation in both colour pattern and vertebral count through the range of *P. elongatus* (Bury 1973, 1999; Nussbaum *et al*. 1983), while geographical variation in *P. stormi* is limited to vertebral count (Brodie 1970; Nussbaum *et al*. 1983).

Several hypotheses have been proposed concerning the evolutionary history of these species, but none have been tested in a molecular framework. *Plethodon elongatus* and *P. stormi* are generally treated as sister species (e.g. Highton 1995; Petranka 1998), however, the geographical pattern of morphological variation has been used to suggest alternative numbers of taxonomic units. The occurrence of morphologically intermediate populations in northern California suggests recent gene flow, supporting treatment of *P. stormi* as a subspecies of *P. elongatus* (Stebbins 2003, and personal communication). Conversely, allopatry and apparent lack of hybridization in Oregon have been used to support continued recognition of two distinct species (Nussbaum *et al*. 1983), with populations in northern California thought to reflect convergent coloration rather than intermixing of the two forms (Brodie 1970; Nussbaum *et al*. 1983). Finally, morphological variation throughout the range of *P. elongatus* may be sufficient to support recognition of additional taxa (Bury 1973, 1999).

Previous systematic research on the *P. elongatus* species group has focused on the initial divergence between the two species. *Plethodon stormi* is thought to be descended from peripheral populations that were isolated from a more widespread ancestral form as a result of climate change. The area of former connection has been postulated to be either in the Applegate River drainage of southern Oregon, or the upper Klamath River region of northern California (Brodie 1970; Bury 1973). These geographical hypotheses imply a phylogeny where *P. stormi* is most closely related to populations of *P. elongatus* in the portion

of the ancestral range from which *P. stormi* 'budded off'. Under either scenario, *P. elongatus* may be paraphyletic with respect to *P. stormi*.

The Klamath-Siskiyou region where these salamanders occur was glaciated at high elevations (Davis 1988; Coleman & Kruckeberg 1999). Cycles of climate change associated with glacial cycles influenced the present distribution of diverse taxa (Soltis *et al*. 1997; Hewitt 2000). Like other species in the genus *Plethodon*, *P. elongatus* and *P. stormi* are fully terrestrial and are sensitive to extremes of temperature and moisture (Welsh & Lind 1995). Range expansion from isolated refugia has been proposed to account for secondary contact between *P. elongatus* and *P. stormi* in the upper Klamath River area (Bury 1973), but the possible role of other demographic events in the relatively recent history of these species has not been explored.

The goal of this study is to use analyses of mtDNA sequences to evaluate previous hypotheses on the history of the *P. elongatus* species group. A combination of phylogenetic and population genetic analyses will be used to examine historical hypotheses at inter- and intraspecific levels and the current taxonomy will be evaluated.

Materials and methods

DNA isolation, amplification and sequencing

Individuals were sampled from throughout the range of both species (Fig. 1, Table 1). Tissue samples consisted of frozen muscle and intestine, or ethanol-preserved tail tips. Frozen samples came from the Museum of Vertebrate Zoology, University of California at Berkeley; most are associated with a preserved voucher specimen (Table 1). Tail tips preserved in 95% ethanol were collected by U.S. Forest Service field crews. These salamanders were released at the capture sites to minimize the impact of collecting on potentially threatened populations (Welsh 1990; Welsh & Lind 1995).

Portions of three mitochondrial protein-coding genes were sequenced. A 385-base-pair (bp) fragment of cytochrome *b* (cyt *b*) was amplified using the primers MVZ15 and cytb2 (Kocher *et al*. 1989; Moritz *et al*. 1992). The primers ND4 and Leu (Arévalo *et al*. 1994) were used to amplify 679 bp of NADH dehydrogenase subunit 4 (ND4). Almost the entire ATPase 6 gene (670 bp) was amplified using primers L9252 and H9923 (Vitt *et al*. 1997). Qiagen DNeasy extraction kits were used to extract whole genomic DNA. The polymerase chain reaction (PCR) was performed using standard components (Palumbi 1996). The PCR thermal profile involved an initial denaturation at 95 °C for 3 min; 36 cycles of denaturation at 95 °C for 1 min, annealing at 45 °C (primers were tested over a range from 42 to 50 °C) for 1 min, and extension at 72 °C for 90 seconds; and a final extension at 72 °C for 2 min.

PCR products were purified using a QIAquick PCR purification kit (Qiagen). They were labelled with fluorescent dye through a cycle-sequencing reaction following standard protocols (Applied Biosystems, Perkin Elmer), and were sequenced using ABI Prism 377 and 3700 automated sequencers with the associated data collection software (Applied Biosystems). Samples were sequenced and read in both primer directions using SEQUENCE NAVIGATOR software (version 1.0.1, Applied Biosystems). Sequences were aligned manually using the amino acid translation as a guide. No insertions or deletions were observed in cyt *b* or ATPase 6. A single nucleotide deletion close to the 3' end of the gene was observed in a subset of the *Plethodon stormi* samples (see Results section). To maintain sequence alignment for these analyses, a single gap was inserted 6 bp from the end of these sequences. ND4 sequences for some samples were available from previous work (Mahoney 2001; Table 1). All sequences used in this study were deposited in GenBank (Table 1).

Phylogenetic and population genetic analyses

Combining all three genes for phylogenetic analysis is preferred because this increases the number of informative nucleotide positions. In addition, the genes are located on the mitochondrion and are not expected to have different histories. However, sequences for all three genes were not available for some individuals, and taxa with large amounts of missing data may have negative effects on phylogenetic analyses (e.g. Wiens & Reeder 1995; Wilkinson 1995). In combined analyses, using maximum parsimony (MP) and maximum likelihood (ML), only samples with sequences for all three gene regions were included to minimize the effect of missing data. The three genes were also analysed separately using minimum evolution (ME) so that every individual was included in at least one phylogenetic analysis. The ME results were used to assign each sample to a major haplotype group, and the MP and ML analyses were used to examine the relationships among haplotype groups.

PAUP* 4.0b10 was used for all phylogenetic analyses (Swofford 2002). The MP, ML and ME analyses were used to examine the phylogenetic relationships among nonidentical haplotypes. These methods were selected because they rely on optimality criteria to select the preferred tree topology, and the methods for ML and ME incorporate models of sequence evolution (Swofford *et al*. 1996). *Plethodon dunni* and *P. vandykei* were used as outgroups in all phylogenetic analyses. Species of *Plethodon* from western North America comprise several highly divergent lineages and these taxa represent two lineages distinct from the *P. elongatus* species group and from each other (Mahoney 2001).

Equal weights were used in MP heuristic searches with 10 random addition replicates. Support was assessed using nonparametric bootstrap with 1000 pseudo-replicates under

Table 1 *Plethodon elongatus* and *P. stormi* sample localities and GenBank accession numbers. Locality numbers as in Fig. 1. Letters indicate multiple samples from single populations. Abbreviations MVZ, Museum of Vertebrate Zoology, UC Berkeley; FC, Frozen Tissue Collection, MVZ; NLS, N. Staub field series. All other samples collected by Forest Service field crews

Population number and sample letter	Haplotype group	Locality	Sample ID/MVZ catalogue number	ND4	ATPase 6	Cytb
1	*elongatus* 1	Oregon; Coos Co.; Eden Ridge, above South Fork Coquille River	N-81006036	AY179220	AY181651	AY183764
2a	*elongatus* 1	Oregon; Josephine Co.; Rainie Falls	43RainieFalls	AY179221	—	AY183765
2b			44RainieFalls	AY179222	AY181652	AY183766
3	*elongatus* 1	Oregon; Josephine Co.; Graves Creek	42GravesCr	AY179223	AY181653	AY183767
4a	*elongatus* 1	Oregon; Curry Co.; Lobster Creek Bridge, north side Rogue River	NLS1621 (Pelo3)	AF329346*	AY181654	AY183768
4b			NLS1623 (Pelo4)	AF329347*	AY181655	AY183769
5	*elongatus* 1	Oregon; Curry Co.; tributary of Lawson Creek	N-10 1024032	AY179224	AY181656	AY183770
6a	*elongatus* 1	Oregon; Curry Co.; Chetco River Road	NLS1636 (Pelo5)	AY179225	AY181657	AY183771
6b			NLS1638 (Pelo6)	—	AY181658	AY183772
7	*elongatus* 1	Oregon; Curry Co.; Bear Creek, close to California border	N-91006028	AY179226	AY181659	AY183773
8a	*elongatus* 1	Oregon; Josephine Co.; 10.4 miles east-southeast Cave Junction, Forest Hwy 3905	MVZ 169014	AY179227	—	AY183774
8b			MVZ 169015	AY179228	AY181660	AY183775
8c			MVZ 169016	AY179229		AY183776
9a	*elongatus* 1	Oregon; Josephine Co.; Oregon Hwy 46, 13.9 miles east Hwy 199 at Cave Junction	MVZ 181547	AY179230	AY181661	AY183777
9b			MVZ 181550	AY179231	—	AY183778
9c			MVZ 181551	AY179232	—	AY183779
10	*stormi*	Oregon; Jackson Co.; Little Humpy Creek	Little Humpy	AY179233	AY181662	AY183780
11	*stormi*	Oregon; Jackson Co.; Ferris Gulch	Ferris Gulch	AY179234	AY181663	AY183781
12	*stormi*	Oregon; Jackson Co.; Nine Mile Creek	Nine Mile	AY179235	AY181664	AY183782
13	*stormi*	Oregon; Jackson Co.; Hinkle Gulch	Hinkle Gulch	AY179236	AY181665	AY183783
14	*stormi*	Oregon; Jackson Co.; Grouse Creek	Grouse Creek	AY179237	AY181666	AY183784
15	*stormi*	Oregon; Jackson Co.; Upper Hanley Gulch Road	MVZ 189117	—	AY181667	AY183785
16	*stormi*	Oregon; Jackson Co.; Carberry Creek	Carberry	AY179238	AY181668	AY183786
17	*stormi*	Oregon; Jackson Co.; Yellowjacket Spring	Yellow Jacket	AY179239	AY181669	AY183787
18	*elongatus* 1	California; Del Norte Co.; Rowdy Creek, near Oregon border	6RowdyCr (SR136)	AY179240	AY181670	AY183788
19	*elongatus* 1	California; Del Norte Co.; North Fork Smith River	21SR130	AY179241	AY181671	AY183789
20a	*elongatus* 1	California; Del Norte Co.; Rowdy Creek Road	MVZ 158704	—	—	AY183790
20c			MVZ 158705	—	—	AY183791
21	*elongatus* 1	California; Del Norte Co.; Jedediah Smith State Park	MVZ 208468	AY179242	AY181672	AY183792
22	*elongatus* 1	California; Del Norte Co.; Myrtle Creek, near Smith River	28nearSR117	AY179243	AY181673	AY183793
23a	*elongatus* 1	California; Del Norte Co.; 1 mile up French Hill Road	MVZ 211852	—	AY181674	AY183794
23b			MVZ 211853	AY179244	AY181675	AY183795
24	*elongatus* 1	California; Del Norte Co.; Craig's Creek, near Smith River	2CraigsCr	AY179245	AY181676	AY183796
25	*elongatus* 1	California; Del Norte Co.; near Gasquet, Middle Fork Smith River	20SR119	AY179246	AY181677	AY183797
26a	*elongatus* 1	California; Del Norte Co.; Miller Redwood Co., along Fall Creek	MVZ 220001	AY179247	—	AY183798
26b		California; Del Norte Co.; Miller Redwood Co., along Rock Creek Road	MVZ 220003	AY179248	AY181678	AY183799
27	*elongatus* 1	California; Del Norte Co.; Hurdygurdy Creek	MVZ 191674	AY179249	AY181679	AY183800
28	*elongatus* 1	California; Del Norte Co.; Jones Ridge, above Jones Creek	17SR105	AY179250	AY181680	AY183801
29	*elongatus* 1	California; Del Norte Co.; Buck Mountain, South Fork Smith River	16SR99	AY179251	AY181681	AY183802
30a	*elongatus* 1	California; Del Norte Co.; Patrick's Creek Road	MVZ 189100	AY179252	AY181682	AY183803
30b		California; Del Norte Co.; Patrick's Creek	FC11455	AY179253	AY181683	AY183804

Table 1 *Continued*

Population number and sample letter	Haplotype group	Locality	Sample ID/MVZ catalogue number	ND4	ATPase 6	Cytb
30c			40PatCr	AY179254	AY181684	AY183805
30d			41PatCr	AY179255	AY181685	AY183806
31	*elongatus* 1	California; Del Norte Co.; Washington Flat, Middle Fork Smith River	4SR125	AY179256	AY181686	AY183807
32	*elongatus* 1	California; Del Norte Co.; Knopti Creek Road	MVZ 189114	AY179257	AY181687	AY183808
33a	*elongatus* 1	California; Del Norte Co.; Elk Creek	MVZ 189128	—	—	AY183809
33b		California; Del Norte Co.; Hwy 99, Stateline Rest Area	36restarea	AY179258	AY181688	AY183810
33c			37restarea	AY179259	AY181689	AY183811
34	*elongatus* 1	California; Del Norte Co.; near Bear Basin Butte, South Siskiyou Fork Smith River	27nearSR112	AY179260	AY181690	AY183812
35	*stormi*	California; Siskiyou Co.; East Fork Indian Creek	FIA14	—	AY181691	AY183813
36	*stormi*	California; Siskiyou Co.; Thompson Creek	FIA16	—	AY181692	AY183814
37a	*stormi*	California; Siskiyou Co.; 0.9 miles (rd) on 1055 above Hutton Campground	MVZ 181523	—	—	AY183815
37b			MVZ 181524	AF329349*	AY181693	AY183816
38	*stormi*	California; Siskiyou Co.; Joe Creek	Joe Creek	AY179261	AY181694	AY183817
39	*stormi*	California; Siskiyou Co.; Elliot Creek	Elliot Creek	AY179262	AY181695	AY183818
40	*stormi*	California; Siskiyou Co.; Slater Butte lookout; past Cade Mountain near Happy Camp	MVZ 211843	—	AY181696	AY183819
41a	*stormi*	California; Siskiyou Co.; 2.4 miles up USFS Road 19-N-01, near Happy Camp, Cade Mountain	MVZ 213112	AY179263	AY181697	AY183820
41b			MVZ 213113	—	AY181698	AY183821
41c			MVZ 214261	AY179264	AY181699	AY183822
41d			MVZ 211845	AY179265	AY181700	AY183823
41e			MVZ 211846	AY179266	AY181701	AY183824
42a	*stormi*	California; Siskiyou Co.; Seattle Creek	38SeattleCr	AY179267	AY181702	AY183825
42b			39SeattleCr	AY179268	AY181703	AY183826
43	*stormi*	California; Siskiyou Co.; 1.7 miles on Forest Road 19N01, north Hwy 96	MVZ 220489	AY179269	AY181704	AY183827
44	*elongatus* 1	California; Siskiyou Co.; small tributary of Grider Creek	KL59no2	AY179270	AY181705	AY183828
45	*elongatus* 1	California; Siskiyou Co.; Walker Creek	8WalkerCr	AY179271	AY181706	AY183829
46a	*elongatus* 1	California; Siskiyou Co.; Little Grider Creek	MVZ 220005	AY179272	AY181707	AY183830
46b			MVZ 220006	AY179273	AY181708	AY183831
47a	*elongatus* 1	California; Siskiyou Co.; 1.9 miles in on USFS Road 15N32 and 16N30	MVZ 208469	AY179274	AY181709	AY183832
47b			MVZ 208471	AY179275	AY181710	AY183833
48a	*elongatus* 1	California; Siskiyou Co.; Wingate Creek	MVZ 220491	AY179276	—	AY183834
48b			MVZ 220492	AY179277	AY181711	AY183835
49a	*elongatus* 1	California; Siskiyou Co.; 0.5 miles south Happy Camp on Elk Creek Road	MVZ 208484	—	—	AY183836
49b			MVZ 208486	—	AY181712	AY183837
50	*elongatus* 1	California; Siskiyou Co.; South Fork China Creek, West of Grider Ridge	13nearKL62	AY179278	AY181713	AY183838
51	*elongatus* 1	California; Siskiyou Co.; Clear Creek Trailhead	1ClearCrTH	AY179279	AY181714	AY183839
52a	*elongatus* 1	California; Siskiyou Co.; Swillup Creek	18KL106	AY179280	AY181715	AY183840
52b		California; Siskiyou Co.; near Swillup Creek	26nearKL106	AY179281	AY181716	AY183841
53	*elongatus* 1	California; Siskiyou Co.; Titus Ridge, above Elk Creek	19nearKL114	AY179282	AY181717	AY183842
54a	*elongatus* 1	California; Del Norte Co.; Wilson Creek Road	MVZ 189019	AY179283	AY181718	AY183843
54b			MVZ 189021	AY179284	—	AY183844
54c			MVZ 189025	AY179285	AY181719	AY183845
55	*elongatus* 1	California; Del Norte Co.; High Prairie Creek	15SR97	AY179286	AY181720	AY183846
56	*elongatus* 3	California; Del Norte Co.; Alder Camp Road	5Requa	AY179287	—	AY183847
57	*elongatus* 1	California; Del Norte Co.; Klamath NF near Dillon Camp	FIA137	AY179288	AY181721	AY183848
58	*elongatus* 1	California; Del Norte Co.; North Fork Bluff Creek	FIA87	AY179289	AY181722	AY183849

Table 1 *Continued*

Population number and sample letter	Haplotype group	Locality	Sample ID/MVZ catalogue number	ND4	ATPase 6	Cytb
59a	*elongatus* 2	California; Del Norte Co.; near Lonesome Ridge, Six Rivers NF	FIA85	AY179290	AY181723	AY183850
59b			N-3 FIA85	AY179291	AY181724	AY183851
59c			N-5 FIA85	AY179292	AY181725	AY183852
60a	*elongatus* 3	California; Del Norte Co.; near Blue Creek Mountain, Six Rivers NF	FIA82	AY179293	AY181726	AY183853
60b			N-6 FIA82	AY179294	AY181727	AY183854
61a	*elongatus* 1	California; Siskiyou Co.; Ti Creek, west Klamath River	22KL146A	AY179295	AY181728	AY183855
61b			23KL146B	AY179296	AY181729	AY183856
62	*elongatus* 1	California; Siskiyou Co.; Little Sandy Bar	MVZ 181599	AY179297	AY181730	AY183857
63	*elongatus* 1	California; Siskiyou Co.; Sandy Bar Creek	24KL153	AY179298	AY181731	AY183858
64	*elongatus* 1	California; Siskiyou Co.; 13.9 miles up USFS Road 15N01 (Orleans); then ≈ 1 mile up USFS Road 15N01F	MVZ 208491	AY179299	AY181732	AY183859
65a	*elongatus* 1	California; Siskiyou Co.; Aubrey Creek, Six Rivers NF	FIA118	AY179300	AY181733	AY183860
65b			N-4 FIA118	AY179301	AY181734	AY183861
66a	*elongatus* 1	California; Siskiyou Co.; near Green Riffle	MVZ 223134	AF329345*	AY181735	AY183862
66b			MVZ 223135	AY179302	AY181736	AY183863
67	*elongatus* 1	California; Siskiyou Co.; Independence River Across, Klamath NF	FIA122	AY179303	AY181737	AY183864
68	*elongatus* 1	California; Siskiyou Co.; Teneyck Creek, northwest of Somes Bar	25KL175	AY179304	AY181738	AY183865
69a	*elongatus* 2	California; Humboldt Co.; Ridge above Camp Creek Six Rivers N. F.	FIA79	AY179305	AY181739	AY183866
69b			N-1 FIA79	AY179306	AY181740	AY183867
70	*elongatus* 2	California; Humboldt Co.; Slate Creek	14SR78	AY179307	AY181741	AY183868
71	*elongatus* 1	California; Humboldt Co.; north Orleans, west Klamath River	3Donahue	AY179308	AY181742	AY183869
72a	*elongatus* 1	California; Siskiyou Co.; Sawyer's Bar, Klamath NF	FIA193	AY179309	AY181743	AY183870
72b			N-2 FIA193	AY179310	AY181744	AY183871
73	*elongatus* 3	California; Humboldt Co.; Tish Tang	9Tishtang	AY179311	AY181745	AY183872
74	*elongatus* 3	California; Humboldt Co.; Horse Linto	MVZ 181602	—	AY181746	AY183873
75	*elongatus* 3	California; Humboldt Co.; Lone Pine Ridge, above Horse Linto Creek	12SR58	AY179312	AY181747	AY183874
76a	*elongatus* 3	California; Humboldt Co.; Waterman Ridge	10WtrmnRdg	AY179313	AY181748	AY183875
76b		California; Humboldt Co.; Waterman Ridge clearcut	11wtrmnrdgCC	AY179314	AY181749	AY183876
77	*elongatus* 3	California; Humboldt Co.; East Fork Willow Creek Campground	EFKCG	AY179315	AY181750	AY183877
78a	*elongatus* 3	California; Humboldt Co.; Friday Ridge, above East Fork Willow Creek	6NO88	AY179316	AY181751	AY183878
78b			N-11 6N088	AY179317	AY181752	AY183879
79a	*elongatus* 3	California; Humboldt Co.; Six Rivers NF	FIA45	AY179318	AY181753	AY183880
79b			N-7 FIA45	AY179319	AY181754	AY183881
80a	*elongatus* 3	California; Trinity Co.; Hwy. 299, rest area 2.6 miles by road southeast of Salyer	MVZ 161872	AY179320	AY181755	AY183882
80b			MVZ 161873	AY179321	AY181756	AY183883
80c			MVZ 215846	—	—	AY183884
81	sp.	California; Siskiyou Co.; Singleton Creek, near Mill Creek	7ScottRvRd	AY179322	AY181757	AY183885
P. dunni				AF370018*	AY181650	AY183763
P. vandykei				AF370021*	AY181649	AY183762

*GenBank accession numbers are taken from Mahoney (2001).

the fast-heuristic option (Felsenstein 1985). Reduced-effort bootstrap searches like the fast-heuristic method have been shown in simulations to have minimal effect on bootstrap values of strongly supported clades (DeBry & Olmstead 2000). Decay indices, an additional measure of support (Bremer 1988; Donoghue *et al*. 1992), were calculated using the program AUTODECAY version 4.0.1 (Eriksson 1998). For ML analyses, the program MODELTEST version 3.06 (Posada & Crandall 1998), was used to select the best fit model of sequence evolution using the data set comprised of samples with all three gene regions, considering only non-identical sequences and including outgroup samples. The best fit model was selected using hierarchical likelihood-ratio tests under a mixed χ^2 distribution with a Bonferroni correction applied to account for multiple tests (Posada & Crandall 1998, 2001). Support was assessed using 100 bootstrap pseudo-replicates and the fast-heuristic option.

ML distances were used in ME analyses and MODELTEST was used to select the model of sequence evolution with the best fit to the data as in the ML analyses. For each gene data set, parameters for the likelihood model were based on analysis of unique in-group sequences plus out-groups. Likelihood parameters were used in heuristic ME searches with 10 random addition replicates. Non-parametric bootstrap using the fast-heuristic option with 1000 pseudo-replicates was used to assess support for the ME topology.

The main haplotype groups recovered by the phylogenetic analyses occupied nonoverlapping geographical areas (see Results) and were treated as separate units in analyses of regional genetic diversity and demographic history (Matocq 2002). As in the phylogenetic analyses, to maximize information from all individuals sequenced, genes were analysed separately and in combination. Variation among DNA sequences (i.e. nucleotide diversity, θ) can be calculated using the average number of pairwise nucleotide differences (π) or the number of polymorphic (segregating) sites (S). Nucleotide diversity calculated as θ_π is sensitive to haplotype frequency, while θ_S is not (Tajima 1989b). Assuming neutrality, the difference between these two measures, known as Tajima's *D*-statistic, can be used to infer demographic history (Tajima 1989a,b). In populations that have remained stable in size over time the two values are expected to be similar, and the *D*-statistic to be close to zero (Tajima 1989b, 1993). Significant values of *D*, either negative or positive, allow one to reject the null hypothesis of population stability. A negative value ($\theta_\pi < \theta_S$) is predicted in populations that have undergone recent increases in size because rare alleles are more abundant than expected. Positive values of *D* suggest elimination of rare alleles which might follow a population bottleneck (Tajima 1989a, 1993). Values of θ_π, θ_S and *D* were calculated using the program ARLEQUIN 2.0 (Schneider *et al*. 2000). The significance of the *D*-statistic was tested by simulating a distribution (1000 replicates) of *D*-values under the null hypothesis of population stability (Schneider *et al*. 2000).

Histograms of the frequency of observed pairwise differences among sequences were plotted using ARLEQUIN, and the shape of the observed mismatch distribution was tested against the null hypothesis of population expansion (Slatkin & Hudson 1991). Mismatch distributions were plotted for all samples, including identical haplotypes, from each haplotype group. Populations which have gone through a period of expansion or growth are expected to have a star-like phylogeny and a unimodal shape to the mismatch distribution reflecting similarity in the amount of divergence among all pairs of haplotypes (Slatkin & Hudson 1991; Rogers & Harpending 1992). Populations which have been stable over time are predicted to have a more balanced phylogeny shape and a bi- or multimodal mismatch distribution (Slatkin & Hudson 1991). The fit between the observed and expected distributions was tested using the sum of squared deviations (Schneider & Excoffier 1999).

Results

One hundred and seven samples from 73 populations of *Plethodon elongatus* and *P. stormi* were sequenced for the ND4 gene (Table 1). Seventy-six unique haplotypes were recovered. Identical haplotypes were usually located in the same population (15 populations), and haplotypes shared across populations (nine instances) were from geographically proximate localities in the same river drainage. Total fragment length was 679 bp, and average sequence length obtained was 674 bp. The best fit model of sequence evolution was the HKY model (Hasegawa *et al*. 1985), incorporating a gamma shape distribution for variable sites and proportion of invariant sites (HKY + G + I). Likelihood parameters fixed for ME analysis (in PAUP command convention) were: Base = (0.3519 0.2638 0.0932); Nst = 2; Tratio = 9.2370; Rates = gamma; Shape = 0.7904; Pinvar = 0.4424.

Six ND4 haplotypes had a single nucleotide deletion compared to the other sequences. In these haplotypes a first position transition from guanine to adenine caused an inferred change from a glycine codon (GGG) to a stop codon (AGG) three codons from the end of the gene (not counting the stop codon which is generated through polyadenylation of the terminal T during transcription yielding TAA; Roe *et al*. 1985). Based on the inferred amino acid sequence, the single nucleotide deletion was downstream from the 'new' stop codon and probably did not cause a deleterious frame shift. The six haplotypes were observed in 10 individuals from the Applegate River drainage in Oregon and the extreme northern edge of California (populations 10–14, 16, 17 and 37–39). Two additional haplotypes, observed in six individuals from Josephine County, OR (populations 8 and 9), displayed the same substitution, resulting in a premature stop codon, but did not have a

downstream deletion. The substitutions resulting in stop codons were inferred to be independent events because the haplotypes did not group together in phylogenetic analyses (below). Premature stop codons, with and without downstream deletions, have been observed in the terminal (3′) region of ND4 in related salamander lineages (Mahoney 2001).

One hundred and seven individuals from 77 populations were sequenced for ATPase 6, yielding 88 unique haplotypes. Identical haplotypes were found in samples from 10 populations and two haplotypes were shared across populations. Fragment length was 670 bp, and average sequence length obtained was 665 bp. The best-fit likelihood model of sequence evolution was the TVM variant of the general time-reversible model (Posada & Crandall 1998, 2001), incorporating gamma (GTR + G) with parameters: Base = (0.3599 0.2299 0.1009); Nst = 6; Rmat = (2.7657 19.5963 1.0461 0.2594 19.5963); Rates = gamma; Shape = 0.3452; Pinvar = 0.

One hundred and twenty-two individuals from 81 populations were sequenced for the cyt *b* fragment, yielding 76 unique haplotypes. Identical haplotypes were found in 18 populations and 12 haplotypes were shared across populations. Average fragment length obtained for the 385-bp region was 383 bp. The best-fit likelihood model of sequence evolution was the TrN variant of GTR, incorporating gamma and proportion of invariant sites (GTR + G + I). Model parameters were Base = (0.3446 0.2113 0.1167); Nst = 6; Rmat = (1.0000 12.0224 1.0000 1.0000 28.9082); Rates = gamma; Shape = 0.6691; Pinvar = 0.5161.

Sequences of all three gene regions were obtained for 98 individuals representing 71 populations. Comparison of the concatenated gene sequences yielded 85 unique haplotypes. The best fit model of sequence evolution for the combined data set was the TVM variant of GTR, incorporating gamma and proportion invariant sites (GTR + *G* + I). Parameters were Base = (0.3519 0.2397 0.0914); Nst = 6; Rmat = (2.3563 24.5216 0.8251 0.4851 24.5216); Rates = gamma; Shape = 0.7937; Pinvar = 0.4203. The model selected for ATPase 6 is nested within this model (i.e. it is a special case of the more general model).

Phylogenetic analyses

All phylogenetic analyses, separate and combined, yielded four genetically distinct, parapatric haplotype groups within the range of *Plethodon elongatus* and *P. stormi*. Figure 1 (see also Table 1) summarizes the population content and geographical distribution of the haplotype groups based on the results of the separate and combined analyses. One haplotype group is comprised of samples from the range of *P. stormi* in the Applegate River drainage in Jackson County, OR, and the border of California (populations 10–17 in OR, and 37–39 in CA) and upper Klamath River, Siskiyou County, CA (populations 35, 36, 40–43). The remaining three haplotype groups, numbered from north to south, comprise samples from the range of *P. elongatus*. Group 1 *P. elongatus* spans most of the range of *P. elongatus* from Coos, Josephine and Curry Counties, OR (populations 1–9), to the northwestern corner of California including the Smith River drainage (populations 18–34), coastal (populations 54, 55) and inland (populations 57, 58) Del Norte County, western Siskiyou County (populations 44–53, 61–68, 72), and northeastern Humboldt County (population 71). Group 2 *P. elongatus* is narrowly distributed in extreme southeastern Del Norte County and northeastern Humboldt Counties (populations 59, 69–70). Group 3 *P. elongatus* covers the southern portion of the range of *P. elongatus*, from coastal Humboldt County (population 56) inland to southern Del Norte County (population 60) and along the Trinity River in Humboldt and Trinity Counties (populations 73–80).

One hundred and sixteen of the one hundred and twenty-two samples were sequenced for two or three gene regions (Table 1), and haplotype group membership was the same in separate analysis of genes. In populations with more than one individual sampled, sympatry of different haplotype groups was not observed. Separate ME and combined MP and ML analyses differed in the relationships among haplotype groups and the degree of support for the haplotype groups. Detailed results for relationships within and among haplotype groups will be presented only for the combined analyses, while results for the genes analysed separately cover the main haplotype groups for comparison with the combined analyses.

A single sample from the eastern margin of the species range in Siskiyou County, CA (population 81, Fig. 1), is the sister to all remaining in-group samples in the phylogenetic analyses and is not assigned to one of the four main haplotype groups. Analyses of ND4 including additional taxa, including species of *Plethodon* from eastern and western North America, *Aneides*, and *Ensatina* (results not shown), support the relationship of this sample as the sister of the remaining samples from the *P. elongatus* species group.

The combined gene data set of 85 haplotypes was 1734 bp, of which 1007 bp were constant, 251 bp were variable but parsimony-uninformative, and 476 bp were parsimony-informative. The ND4 region was 679 bp, of which 407 bp were constant, 89 bp were variable but parsimony-uninformative and 183 bp were parsimony-informative. The ATPase 6 region was 670 bp with 366 bp constant, 112 bp variable but uninformative and 192 bp parsimony informative. The cyt *b* region was 385 bp, with 234 bp constant, 50 bp variable but uninformative and 101 bp parsimony-informative.

MP analysis of the combined gene data set resulted in 1924 most parsimonious trees (MPTs; Fig. 2). Tree length was 1722 steps, consistency index (CI) was 0.518, and

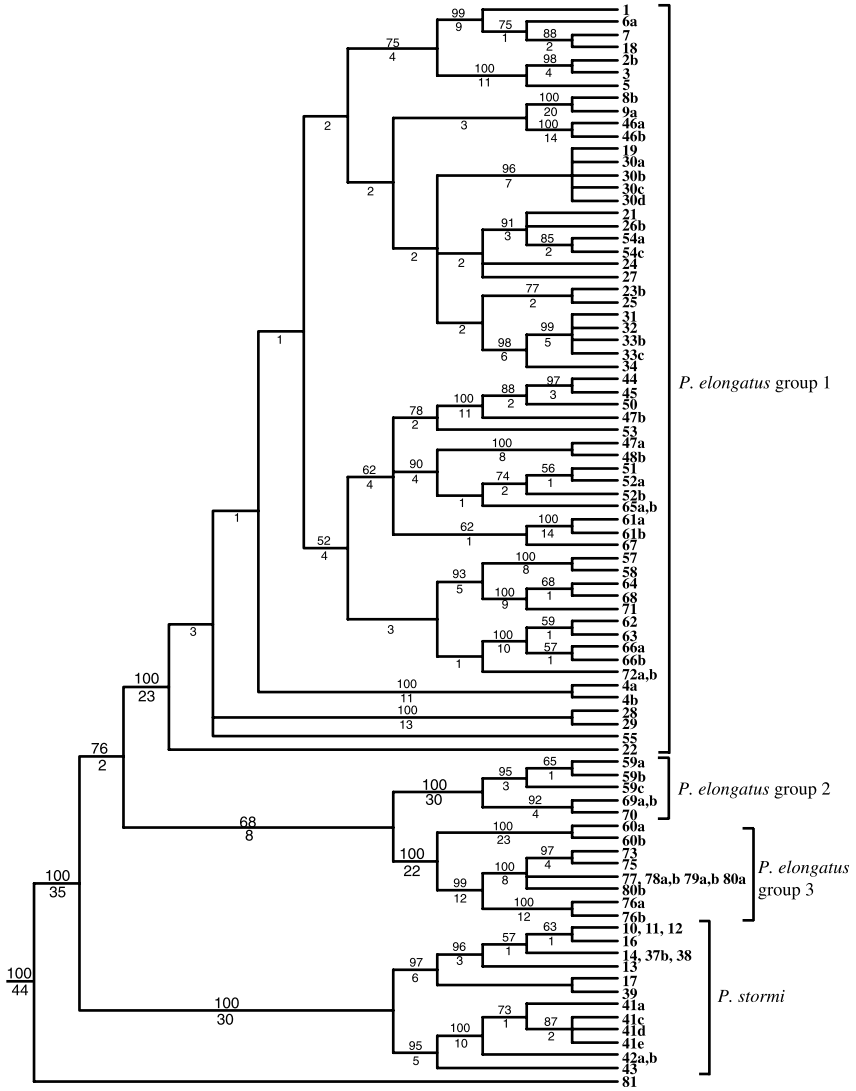

Fig. 2 Results of maximum parsimony analysis of samples with all three mitochondrial gene regions. Strict consensus topology of 1924 MPTs, length 1722 steps (CI 0.518, RI 0.826). Bootstrap support values greater than 50% above branches, decay indices below branches. Tree is outgroup rooted (outgroups not shown). Population and sample numbers as in Table 1.

retention index (RI) was 0.826. Monophyly of the ingroup was highly supported (100% bootstrap; 44 decay index, DI), as was the sister group relationship of population 81 to the remaining ingroup samples (100%; 35 DI). Each of the four haplotype groups was strongly supported in terms of bootstrap and DI values: *P. stormi* 100%, 30 DI; Group 1 *P. elongatus* 100%, 23 DI; Group 2 *P. elongatus* 100%, 30 DI; Group 3 *P. elongatus* 100%, 22 DI. Relationships among the haplotype groups had moderate support. *Plethodon stormi* is the sister group of the three *P. elongatus* haplotype groups which formed a clade (76%, 10 DI). Group 2 and Group 3 *P. elongatus* were sister clades (68% bootstrap, 8 DI), and this pair was the sister of Group 1 *P. elongatus*.

Much of the basal structure within Group 1 *P. elongatus* had less than 50% bootstrap values (Fig. 2). A few groups comprised of multiple haplotypes had moderate to strong support, for example, samples from the North Fork of the Smith River drainage (populations 31–34; 98%) and Klamath River drainage (47, 48, 51, 52, 65; 90%; and 57, 58, 64, 68, 71; 90%). Within Group 3 *P. elongatus*, the population from the Klamath River drainage (population 60) was sister to the samples from the Trinity River drainage (populations 73, 75–80). The Trinity River drainage group had high bootstrap support (99%). A basal split was recovered for the *P. stormi* haplotype group. Bootstrap support was high for clades comprised of Applegate River drainage samples (populations 10–14, 16, 17, 37–39; 97%) and upper Klamath River samples (populations 41–43; 95%).

Maximum likelihood analysis resulted in a single tree, likelihood score — 10780.62554 (Fig. 3), with the same relationships among the main haplotype groups as in MP analysis. In the ML analysis, as in the MP analysis, the four main haplotype groups were strongly supported, but relationships among haplotype groups were weakly supported. The *P. stormi* haplotype group (95%) was the sister to a clade comprised of the three *P. elongatus* haplotype

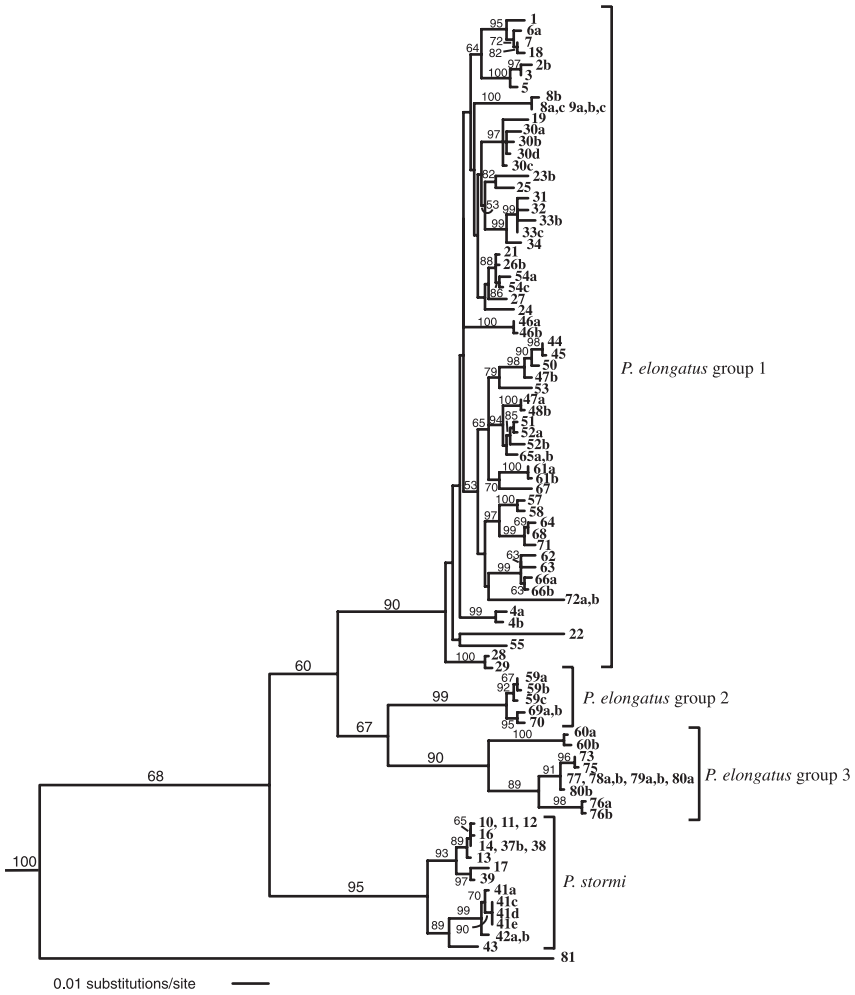

Fig. 3 Results of maximum likelihood analysis of samples with all three mitochondrial gene regions. Single ML tree, –ln 10780.62554. Bootstrap support values greater than 50% are indicated on branches. Tree is outgroup rooted (outgroups not shown). Population and sample numbers as in Table 1.

groups. Groups 2 and 3 *P. elongatus* received strong support (99% and 90%, respectively) but the sister group relationship was weakly supported (67%), as was the monophyly of the three *P. elongatus* haplotype groups (60%). One difference between MP and ML analyses was the support for monophyly of in-group samples exclusive of population 81. This grouping had high bootstrap support in MP analysis (100%) but only weak support in ML analysis (68%).

Analysis of the ND4 region yielded a single ME tree, score 1.90052 (Fig. 4). All samples of *P. elongatus* and *P. stormi* formed a monophyletic group with high bootstrap support (98%). Monophyly of the in-group samples, with respect to population 81 was weakly supported (58%), and there was less than 50% bootstrap support for relationships among the four haplotype groups. Bootstrap support for the main haplotype groups was weak to moderate (57–89%), and lower than in the combined analyses (Figs 2 and 3).

In ME analysis of ATPase 6 (not shown), *P. stormi* clustered as the sister group of Group 1 *P. elongatus*, with Group 3 *P. elongatus* and Group 2 *P. elongatus* sequentially basal. The topology was similar to that found with ND4, but the positions of Group 2 and 3 *P. elongatus* were reversed. In analysis of cyt *b*, Group 2 and Group 3 *P. elongatus* were sister groups and Group 1 *P. elongatus* was the sister of this pair. *Plethodon stormi* joined as the sister to a clade comprised of the three *P. elongatus* haplotype groups. These were the same relationships as those recovered in combined analyses. Compared with analyses of ND4, bootstrap support values for the main haplotype groups were higher in analyses of ATPase6 and lower in analyses of cyt *b*. For both ATPase 6 and cyt *b*, as seen with ND4, there was less than 50% bootstrap support for relationships among the four main haplotype groups.

Support values for relationships among the major haplotype groups were higher in the combined analysis than in analyses of the genes separately and a sister group relationship between *P. elongatus* and *P. stormi* is the preferred phylogenetic hypothesis based on this result. The number of OTUs, operational taxonomic units (i.e. unique haplotypes) included in the combined analysis ($n = 85$) was similar to or greater than the number in the separate analyses

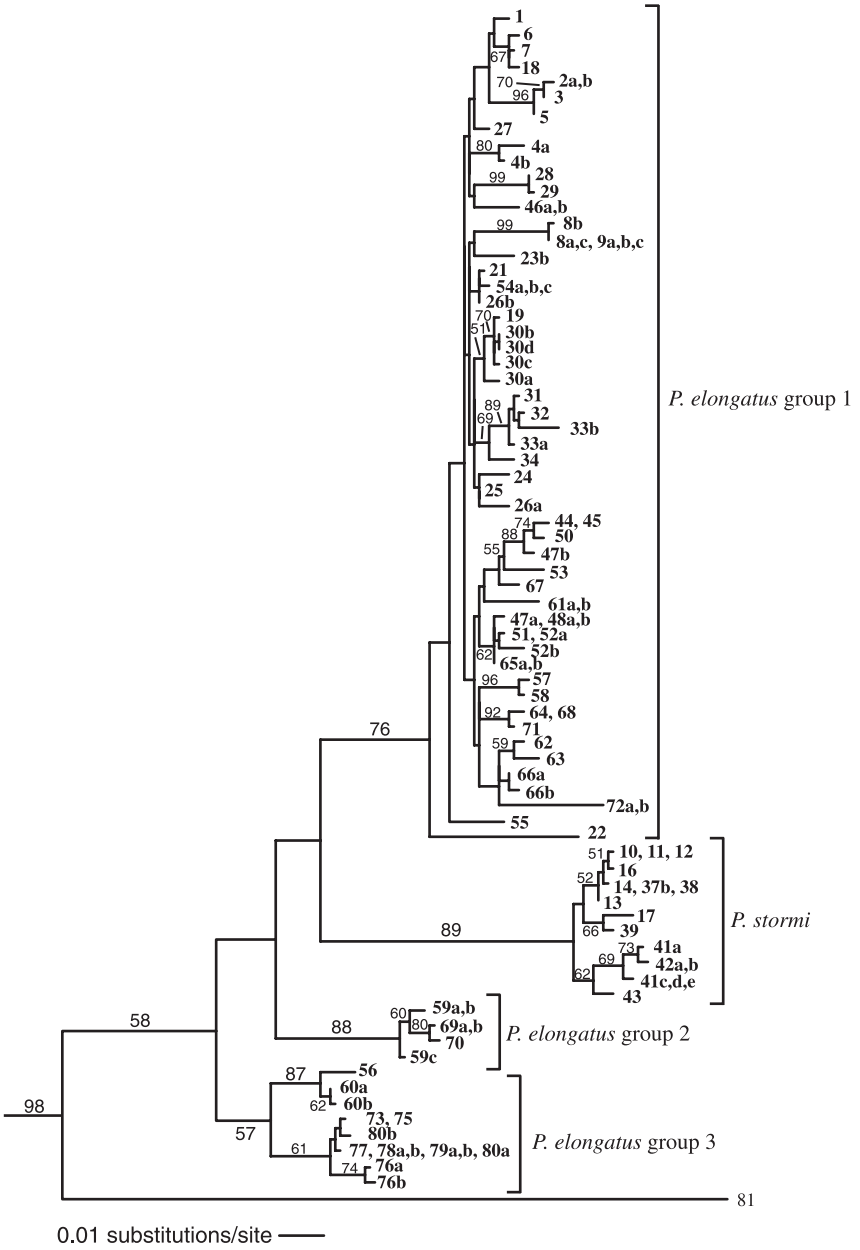

Fig. 4 Minimum evolution analysis of ND4 sequences. Single ME tree, score 1.90052. Non-parametric bootstrap support values above 50% are shown on branches. Tree is out-group rooted (out-groups not shown). Population and sample numbers as in Table 1.

(ND4: 76; ATPase 6: 88; cyt *b*, 76), so the increase in bootstrap support values is probably the result of the increase in the amount of sequence data relative to the number of OTUs.

Genetic diversity and regional demographic history

Maximum sequence divergence of ND4 sequences within haplotype groups ranged from 1% (Group 2 *P. elongatus*) to 5.7% (Group 1 *P. elongatus*; Table 2). Maximum divergence within *P. stormi* was similar to maximum divergence within Group 3 *P. elongatus*. Average per cent sequence divergences among the three *P. elongatus* haplotype groups ranged from 6.1% to 6.9%. The three groups had similar amounts of divergence from the *P. stormi* haplotype group, ranging from 7.5% to 8.5%. Considering all *P. elongatus* samples together, maximum per cent divergence within the group was 8.4% and the average divergence to *P. stormi* was 7.7% (range 6.2–9.2%, Table 2). Per cent divergences for ATPase 6 within and among haplotype groups tend to be 1–2% higher than comparisons among ND4 sequences. The average per cent divergence between *P. elongatus* and *P. stormi* for ATPase was 9.5% (range 7.5–11.3%; Table 3). Average per cent divergences of cyt *b* among *P. elongatus* haplotype groups was similar to values for ND4. For cyt *b*, the average per cent divergence between the two species was 8.1% (range 6.0–10.7, Table 4).

Table 2 Pairwise differences (uncorrected per cent divergence) within and among haplotype groups for ND4 gene region

	P. elongatus 1	*P. elongatus* 2	*P. elongatus* 3	all *P. elongatus*	*P. stormi*	Population 81	Out-groups
P. elongatus 1	**5.66**	6.51 (5.65–7.53)	6.93 (5.81–8.36)		7.53 (6.22–9.09)	12.38 (11.65–13.22)	20.55 (18.21–22.78)
P. elongatus 2		**1.04**	6.09 (5.51–6.92)		7.89 (7.0–8.8)	13.13 (13.03–13.36)	20.12 (18.81–21.42)
P. elongatus 3			**3.43**		8.49 (7.75–9.17)	12.48 (11.96–13.22)	20.53 (18.81–22.77)
all *P. elongatus*				**8.36**	7.67 (6.22–9.17)	12.44 (11.65–13.36)	20.52 (18.2–22.78)
P. stormi					**2.77**	12.18 (11.8–12.77)	20.67 (18.79–23.07)
Population 81							20.32 (19.59–21.04)

Average (minimum–maximum) per cent divergence among haplotype groups shown above the diagonal. Maximum per cent divergence within haplotype groups shown along the diagonal in bold. *Plethodon elongatus* is represented by three constituent haplotype groups (1 through 3) and as a single group (all *P. elongatus*).

Table 3 Pairwise differences (uncorrected percent divergence) within and among haplotype groups for ATPase 6 gene region. See Table 2 for details

	P. elongatus 1	*P. elongatus* 2	*P. elongatus* 3	All *P. elongatus*	*P. stormi*	Population 81	Out-groups
P. elongatus 1	**4.78**	7.72 (6.57–8.81)	9.05 (7.76–10.85)		9.46 (7.46–11.34)	12.42 (11.19–13.43)	23.22 (21.79–24.92)
P. elongatus 2		**0.9**	6.84 (6.42–7.3)		9.06 (8.06–9.85)	11.64 (11.34–11.94)	21.79 (21.49–22.09)
P. elongatus 3			**5.22**		9.9 (9.07–11.13)	13.64 (13.28–14.33)	23.03 (21.94–24.33)
all *P. elongatus*				**10.85**	9.49 (7.46–11.34)	12.51 (11.19–14.33)	23.12 (21.49–24.92)
P. stormi					**4.47**	12.83 (12.27–13.28)	23.47 (20.9–24.34)
Population 81							**21.79** (21.49–22.09)

Table 4 Pairwise differences (uncorrected per cent divergence) within and among haplotype groups for cyt *b* gene region. See Table 2 for details

	P. elongatus 1	*P. elongatus* 2	*P. elongatus* 3	All *P. elongatus*	*P. stormi*	Population 81	Out-groups
P. elongatus 1	**5.38**	6.87 (4.42–8.83)	6.7 (4.16–8.83)		8.09 (5.97–10.65)	13.98 (12.23–15.84)	18.44 (16.79–20.36)
P. elongatus 2		**1.56**	6.56 (5.71–7.27)		8.07 (6.66–9.35)	15.58 (15.32–15.84)	17.86 (17.4–18.44)
P. elongatus 3			**4.16**		8.61 (7.27–9.61)	15.5 (15.32–15.6)	19.08 (17.66–20.78)
All *P. elongatus*				**8.83**	8.14 (5.97–10.65)	14.21 (12.23–15.84)	18.46 (16.79–20.78)
P. stormi					**4.16**	14.73 (14.03–15.3)	18.18 (16.62–19.22)
Population 81							18.96 (17.66–20.26)

Table 5 Genetic diversity and results of population genetic tests

Haplotype group	n	n_H	θ_S (SD)	θ_π (SD)	Tajima's D	P(D simul. < D obs.)	SSD	P(Sim. Ssd ≥ Obs. Ssd)
ND4								
P. elongatus group 1	69	53	25.19 (6.84)	14.82 (7.44)	–1.41696	**0.043**	0.00181757	0.369
P. elongatus group 3	14	8	10.06 (4.04)	9.32 (5.12)	–0.31822	0.408	0.04977291	0.37
P. stormi	17	10	7.69 (3.03)	8.43 (4.59)	0.38621	0.704	0.04512405	0.077
ATPase 6								
P. elongatus group 1	64	60	29.19 (7.98)	17.15 (8.57)	–1.43625	**0.049**	0.00054468	0.923
P. elongatus group 3	14	8	13.21 (5.20)	12.3 (6.64)	–0.30037	0.425	0.08443857	0.258
P. stormi	22	15	13.99 (4.94)	12.24 (6.42)	–0.49341	0.355	0.02119406	0.237
cyt *b*								
P. elongatus group 1	76	56	14.69 (4.07)	11.46 (5.82)	–0.73433	0.271	0.00326838	**0.037**
P. elongatus group 3	16	6	6.33 (2.59)	6.74 (3.76)	0.26353	0.637	0.08822775	0.052
P. stormi	23	9	5.69 (2.19)	7.47 (4.04)	1.15695	0.906	0.04795574	0.056
complete sequences								
P. elongatus group 1	61	59	69.02 (18.53)	43.32 (21.12)	–1.32198	0.053	0.00098571	0.772
P. elongatus group 3	13	8	27.39 (10.61)	29.24 (15.39)	–0.29215	0.421	0.07200762	0.097
P. stormi	17	12	23.37 (8.52)	27.02 (13.95)	0.66183	0.774	0.05871027	**0.018**

n, number of individuals; n_H, number of haplotypes; SSD, sum of squared deviations of mismatch distribution from prediction under population expansion model. Significant P-values in bold face.

Group 2 *P. elongatus* was excluded from analyses of regional diversity because of the small number of populations and individuals included (three and six, respectively). Values of Tajima's D for Group 1 *P. elongatus* were negative for each gene region, and analyses of ND4 and ATPase 6 were significantly different from zero (Table 5). Values of D were not consistently positive or negative for the other groups and no other haplotype group had a value of D significantly different from zero for any gene region (Table 5). Analysis of the combined gene data set (samples with all three gene regions, including identical sequences) did not find significant deviation from zero for any group, although Group 1 *P. elongatus* approached significance ($P = 0.053$; Table 5).

Group 1 *P. elongatus* had a unimodal mismatch distribution for each gene analysed, while Group 3 *P. elongatus* and *P. stormi* had multimodal distributions. Figure 5 shows the mismatch distributions from ND4 sequence data and the combined gene data set. The mismatch distributions for ATPase 6, cyt *b* (not shown) and the combined data set were visually similar. None of the mismatch distributions for ND4 or ATPase 6 were significantly different from the null expectation under a model of population growth (Table 5; Fig. 5). In analysis of the cyt *b* data set, Group 1 *P. elongatus* was significantly different from the null expectation ($P = 0.037$) and Group 3 *P. elongatus* and *P. stormi* approached significance (Table 5). The combined gene data set had similar patterns, and only the *P. stormi* haplotype group deviated significantly from the null expectation ($P = 0.018$; Table 5, Fig. 5).

Discussion

Phylogenetic history

The current treatment of *Plethodon elongatus* and *P. stormi* as sister species (Highton 1995; Petranka 1998) is at odds with biogeographic hypotheses that imply paraphyly of *P. elongatus* with respect to *P. stormi* (Brodie 1970; Bury 1973, 1999). The phylogenetic predictions from taxonomy and biogeography may be viewed as bounds on a range of possible outcomes as opposed to exclusive alternatives. The results presented here do not unambiguously support either extreme, however, the most well-supported phylogenetic topologies, from the combined MP and ML analyses, support continued recognition of *P. elongatus* and *P. stormi* as reciprocally monophyletic sister species. Relationships and interactions with a possible third species in this group, represented by a single population in this study (population 81, Figs 2 and 3), are being studied in more detail (L. Mead and D. DeGross, personal communication), and will not be discussed in detail here.

In agreement with the current taxonomic arrangement, *P. stormi* was recovered as the sister group of a monophyletic *P. elongatus* in analyses of cyt *b* and the combined analyses (MP and ML). Support in the cyt *b* analyses was weak (less than 50%), and only moderate in the combined analyses (Figs 2 and 3), though greater than in any of the separate analyses. In the ND4 and ATPase 6 analyses, *P. stormi* was sister group of the northernmost haplotype clade within *P. elongatus* (Group 1 *P. elongatus*), though

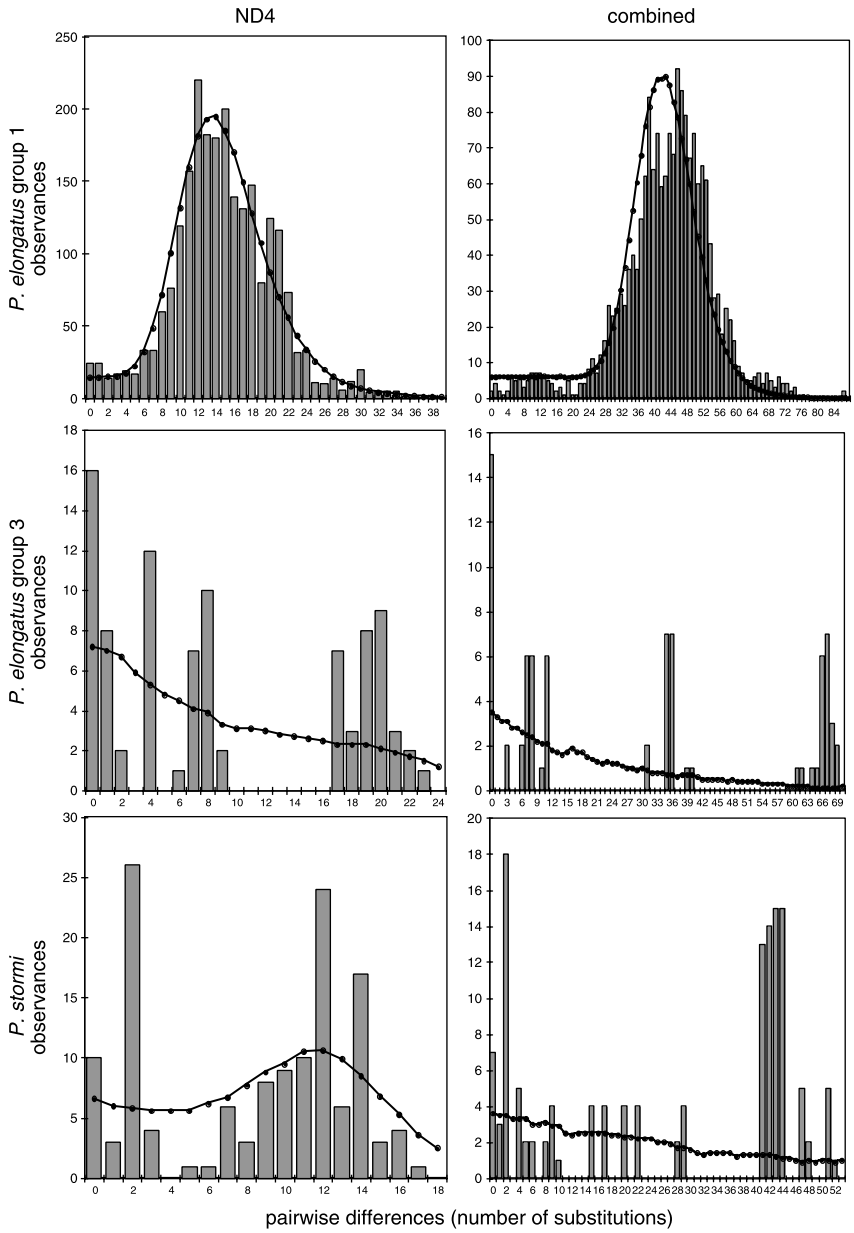

Fig. 5 Mismatch histograms for the ND4 gene region (left column) and the combined gene data set (right column). Observed pairwise substitutional differences (grey boxes) are compared with values simulated under a population expansion model (black line with dots). Details in text.

support in each case was weak (less than 50%). An affinity between *P. stormi* and northern populations of *P. elongatus* is predicted by the biogeographic scenarios, however, at a fine geographical scale, the phylogeny recovered is not in precise agreement with either previously presented hypothesis. Brodie (1970) proposed a connection between *P. stormi* in the Applegate River and nearby *P. elongatus* (populations 8 and 9 in this study; Fig. 1). Bury (1973, 1999) proposed a relationship between *P. stormi* and *P. elongatus* along the Klamath River (e.g. populations 44–50; Fig. 1). Although the *P. elongatus* populations under either hypothesis are in the northernmost *P. elongatus* haplotype group (Group 1), populations in this group are more closely related to each other than to *P. stormi*. It is possible that more recent population history, perhaps subsequent to the last cycle of glaciation, has obscured patterns resulting from earlier phylogenetic divergences within this group (see below).

Demographic history

Although northern California and Oregon had only high elevation glaciers (e.g. Davis 1988; Coleman & Kruckeberg 1999), climate changes correlated with glacial cycles probably influenced distribution of regional faunas (e.g. Soltis *et al*. 1997; Hewitt 2000), including salamanders, which are

sensitive to temperature and moisture regimes (Feder 1983; Welsh & Lind 1995). The Klamath-Siskiyou region occupied by these salamanders has been proposed as a refugial area for many plant taxa (Whittaker 1961; Smith & Sawyer 1988; Soltis *et al*. 1997). Most studies on the influence of glaciation on genetic structure have examined widely ranging species that have moved into previously glaciated areas (e.g. Soltis *et al*. 1997; Conroy & Cook 2000). The *P. elongatus* group shows comparable demographic patterns and genetic structure on a relatively small geographical scale.

Genetic divergences between *P. elongatus* and *P. stormi* are similar to values reported for other Pacific Northwest amphibians, both within and among species. Average per cent divergence between *P. elongatus* and *P. stormi* ranges from 7.67% to 9.49% across the three genes in this study (Tables 2, 3, 4). Mitochondrial DNA divergences among salamanders and frogs in the same region range from 5.5% to 10.1% (*Ensatina* salamanders, Moritz *et al*. 1992; *Taricha* newts, Tan & Wake 1995; *Rana* frogs, Macey *et al*. 2001). Although average divergences among the *P. elongatus* groups are lower than comparisons between *P. elongatus* and *P. stormi*, the ranges of values in these comparisons overlap broadly; many comparisons among *P. elongatus* haplotype groups are similar to those between *P. elongatus* and *P. stormi.* Relatively deep genetic divergences across narrow geographical ranges have been observed in many plethodontid salamanders (e.g. Moritz *et al*. 1992; Chippindale *et al*. 2000; Jockusch & Wake 2002).

Genetic variation in Group 1 *P. elongatus* conforms to predictions based on population expansion models with both Tajima's *D*-statistic and mismatch histograms. Group 1 *P. elongatus* was the only haplotype group with consistently negative values of Tajima's *D*, where a value of zero is predicted with population stability. Values for Group 1 *P. elongatus* were significantly different from zero for two of the three gene regions examined and approached significance when the genes were combined. Negative values of Tajima's *D* result from an excess of rare alleles (i.e. haplotypes), as expected after a recent population expansion (Tajima 1989b, 1993). Complementing the Tajima's *D*-statistic, mismatch histograms for Group 1 *P. elongatus* have the unimodal shape predicted under a model of population expansion (Slatkin & Hudson 1991).

Group 1 *P. elongatus* is significantly different from the null expectation for cyt *b*, yet the histogram visually agrees with the predicted shape under the model of expansion. Although they used a different method of testing deviation from the null expectation, Slatkin & Hudson (1991) presented unimodal histograms that were significantly different from a Poisson distribution, although this was difficult to distinguish visually. In addition, histograms which deviate from a unimodal shape may not always reject the null expectation (Schneider & Excoffier 1999). Mismatch distributions may have value more as heuristic rather than statistical predictors of demographic history.

The population expansion model predicts a star-like phylogeny associated with the unimodal mismatch distribution (Slatkin & Hudson 1991). The phylogenetic topology of Group 1 *P. elongatus* has star-like properties in that many internodes among haplotypes are short and have low character support. This pattern is seen for each individual gene and when all three genes are combined. In addition to poorly supported resolution among haplotypes, the similar length of the branches suggests descent from a single coalescent event at the base of the clade with subsequent divergence among lineages also proposed to correlate with rapid expansion (Slatkin & Hudson 1991).

Group 3 *P. elongatus* and the *P. stormi* haplotype group have similar results under the Tajima's *D*-statistic and mismatch histogram comparisons. Neither group has a consistent pattern of negative or positive values for Tajima's *D*, and these values are not significant for the gene data analysed separately or combined. The mismatch histograms are multimodal for each gene region, though only significantly different from the null expectation for *P. stormi* when the data are combined. The phylogenetic topology of both groups conforms to predictions of long-term population stability resulting in substantial phylogenetic structure (Slatkin & Hudson 1991), and the groups also have a similar geographical distribution of genetic diversity. Each group has a basal split and a geographical area in one subgroup with very low genetic variation, including haplotypes shared among populations. The multimodal mismatch distribution for each group is related to the phylogenetic topology. Comparisons across the basal divergence account for the peak of the highest divergences on the right side of the histogram, and comparisons among the most similar haplotypes account for the peak on the left side of the histogram. Variation within the subgroups (on each side of the basal split) results in the intermediate values in the histogram.

Within Group 3 *P. elongatus*, six individuals from four populations (populations 77–80) in the southern portion of the Trinity River drainage share a single ND4 haplotype, and an additional sample from one population (80) differs by a single substitution (Fig. 2). Eight samples from eight populations (10–14, 16, 37b, 38) of *P. stormi* in the Applegate River drainage have four ND4 haplotypes that differ by one to three substitutions (Fig. 2). Each gene, and the combined analyses, shows a similar pattern for Group 3 *P. elongatus* and *P. stormi*: low variability among most samples from one river drainage. In the ATPase 6 data set, the only two cases of haplotypes shared across populations are six individuals from six populations in the Applegate drainage (populations 10–12, 14, 16, 37, 38) and seven individuals from four populations in the southern Trinity River drainage (populations 77–80). For cyt *b*, 10 individuals

from nine populations in the Applegate drainage (populations 10–16, 37, 38) share a haplotype, as do eight individuals from four populations from the Trinity River drainage (populations 77–80).

A very recent population expansion will result in a peak in the mismatch distribution at the left side of the graph, comprising the comparisons among identical and highly similar sequences (Rogers & Harpending 1992). Although all samples, including identical samples, are included in the mismatch analyses, the left-hand peak in these graphs is not the result of differential sampling among populations. Within *P. stormi* in the Applegate River drainage, all populations but one are represented by single individuals, so identical haplotypes are almost entirely the result of haplotype sharing among populations. Five of 10 populations within Group 3 *P. elongatus* have two or more samples. In two populations more than one haplotype was recovered (60 and 80), and haplotypes are shared among populations, as well as by individuals within some populations. Dividing these two haplotype groups more finely for demographic analysis would result in reduction of the number of included populations and low statistical power, however, the observed patterns of genetic diversity, primarily identical and near-identical haplotypes shared among multiple populations, suggest that Group 3 *P. elongatus* and *P. stormi* have each recently expanded into one area of their geographical range.

Species history

Plethodon elongatus and *P. stormi* contact one another in the upper Klamath River and the morphological transition between forms suggests introgression (Stebbins 2003; Brodie 1970). The mtDNA boundary is broadly congruent with this transition area. The mtDNA break occurs between populations 43 (*P. stormi*) and 46 (*P. elongatus*), in an area where individuals display the *P. elongatus* colour pattern (D. Wake, personal communication). Individuals intermediate in coloration occur to the east at Seattle Creek (population 42; D. Wake, personal communication). Populations 36–39 display *P. stormi* coloration. The relatively narrow morphological transition zone between *P. elongatus* and *P. stormi* and the concordance between morphological and genetic borders, despite apparent gene flow, suggest a relatively recent contact between these lineages.

Genetic subdivision within *P. elongatus* is not concordant with the pattern of morphological variation in this species. Populations of *P. elongatus* from coastal California tend to be darker and smaller with an obscure dorsal stripe, particularly in older individuals, and less white flecking than inland forms along the Klamath River (Stebbins 1951, 2003; Brodie 1970; Nussbaum *et al*. 1983). The transition between coastal and inland colour patterns is clinal, following the Klamath River (Bury 1973, 1999). Populations in Oregon are similar to forms from coastal California (Brodie 1970; Nussbaum *et al*. 1983). In the southern part of the range, inland populations from the Trinity River basin may be morphologically distinct from populations further to the north in the Klamath River (Bury 1999). Mitochondrial data support the affinity of populations in Oregon and California, but there is no genetic break between populations from coastal and inland areas of California. A single haplotype clade occurs in these areas and there is no support for geographical structure within the clade. Genetic breaks in the southern portion of the range of *P. elongatus* are not concordant with the morphological patterns with respect to the distribution of Group 3 *P. elongatus* and the previously unsuspected presence of Group 2 *P. elongatus*. Populations in Group 3 *P. elongatus* are primarily in the Trinity River basin (populations 73–80; Fig. 1), but this clade extends northward and includes populations in coastal (population 56) and inland (population 60; Fig. 1) areas of the Klamath River. Incongruence between genetic and morphological borders, and the broad, clinal nature of morphological transitions suggest a longer period of contact and gene flow among previously discrete lineages within *P. elongatus*, compared with the relatively recent contact between *P. elongatus* and *P. stormi*.

As noted above, the genetic divergences among haplotype groups within *P. elongatus* are nearly as deep as those between *P. elongatus* and *P. stormi.* Although it seems likely that recent gene flow unites these groups, more data are required to assess this possibility. The large divergences among clades and the geographical structure of the mtDNA lineages are an indication of historical isolation of populations within *P. elongatus*. It is recommended that the three main haplotype groups within *P. elongatus* be treated as evolutionarily significant units (ESUs, Moritz 1994a,b) to recognize both the genetic distinctiveness and parapatric geographical distributions of the groups.

Continuing studies on *P. elongatus* will focus in greater detail on the geographical pattern of genetic structure and will examine the boundaries between the major genetic lineages on a finer geographical scale (Mahoney and Welsh, in preparation). The three haplotype groups comprising *P. elongatus* closely approach one another at the three-way border between Del Norte, Siskiyou and Humboldt Counties, CA (Fig. 1). Several populations in this area are represented by more than one sample, and the haplotype groups have not been found in sympatry. The Klamath River separates Groups 1 and 3 at the coast, however, Group 3 is found north of the Klamath River further inland. The type locality of *P. elongatus* is at Requa (Van Denburgh 1916), on the north side of the mouth of the Klamath River. Populations 54 and 55, north of the type locality are in Group 1 *P. elongatus*, while sample 56, from near the coast immediately south of the Klamath River, is in Group 3 *P. elongatus*. Determining the geographical distribution of the

mitochondrial groups will have a bearing on taxonomic decisions if future studies of morphology and nuclear markers find support for dividing *P. elongatus* into more than one species.

Acknowledgements

This study was funded by PNW 00-CR-11261951-194 from USDA/Forest Service (Pacific Northwest Research Station), to M. Mahoney and D. Wake. Many thanks go to D. Olson for giving me the opportunity to work on this project and for her continuing encouragement and enthusiasm. Ethanol-preserved tail-tips were provided by D. Olson, H. Welsh, R. Naumann, L. Ollivier and their hard-working U.S. Forest Service field crews. T. Devitt, S. Kuchta, R. Mueller, L. Rissler, K. Ruegg, V. Vredenburg and D. Wake provided useful comments on draft manuscripts and the project in general. Special thanks also go to D. Frost and W. Wheeler for various types of support during my postdoctoral studies at the American Museum of Natural History, New York. Molecular sequencing was conducted in the Evolutionary Genetics Laboratory, Museum of Vertebrate Zoology, UC Berkeley, and the Molecular Systematics Laboratories, AMNH.

References

Arévalo ES, Davis K, Sites JW Jr (1994) Mitochondrial DNA sequence divergence and phylogenetic relationships among eight chromosome races of the *Sceloporus grammicus* complex (Phrynosomatidae) in central Mexico. *Systematic Biology*, **43**, 387–418.

Avise JC (2000) *Phylogeography: the History and Formation of Species*. Harvard University Press, Cambridge, MA.

Bremer K (1988) The limits of amino acid sequence data in angiosperm phylogenetic reconstruction. *Evolution*, **42**, 795–803.

Brodie ED Jr (1970) Western salamanders of the genus *Plethodon*: systematics and geographic variation. *Herpetologica*, **26**, 468–516.

Brodie ED Jr (1971) *Plethodon stormi* Highton and Brame. Siskiyou Mountains salamander. *Catalogue of American Amphibians and Reptiles*, **103**, 1–2.

Brodie ED Jr, Storm RM (1971) *Plethodon elongatus* Van Denburgh. Del Norte salamander. *Catalogue of American Amphibians and Reptiles*, **102**, 1–2.

Brunsfeld SJ, Sullivan J, Soltis DE, Soltis P (2001) Comparative phylogeography of northwestern North America: a synthesis. In: *Integrating Ecology and Evolution in a Spatial Context* (eds Silvertown J, Antonovics J), pp. 319–339. Blackwell Science, Oxford.

Bury RB (1973) Western *Plethodon*: systematics and biogeographic relationships of the *elongatus* group. *Hiss News-Journal*, **1**, 56–57.

Bury RB (1999) Evolution, zoogeography and management of the Del Norte and Siskiyou Mountain salamanders. *Northwestern Naturalist*, **80**, 121.

Bury RB, Pearl CA (1999) Klamath-Siskiyou herpetofauna: biogeographic patterns and conservation strategies. *Natural Areas Journal*, **19**, 341–350.

Chippindale PT, Price AH, Wiens JJ, Hillis DM (2000) Phylogenetic relationships and systematic revision of central Texas hemidactyliine plethodontid salamanders. *Herpetological Monographs*, **14**, 1–80.

Coleman RG, Kruckeberg AR (1999) Geology and plant life of the Klamath-Siskiyou Mountain region. *Natural Areas Journal*, **19**, 320–340.

Conroy CJ, Cook JA (2000) Phylogeography of a post-glacial colonizer: *Microtus longicaudus* (Rodentia: Muridae). *Molecular Ecology*, **9**, 165–175.

Davis PT (1988) Holocene glacier fluctuations in the American Cordillera. *Quaternary Science Reviews*, **7**, 129–157.

DeBry RW, Olmstead RG (2000) A simulation study of reduced tree-search effort in bootstrap resampling analysis. *Systematic Biology*, **49**, 171–179.

DellaSala DA, Reid SB, Frest TJ, Strittholt JR, Olson DM (1999) A global perspective on the biodiversity of the Klamath-Siskiyou Ecoregion. *Natural Areas Journal*, **19**, 300–319.

Donoghue MJ, Olmstead RG, Smith JF, Palmer JD (1992) Phylogenetic relationships of Dipsacales based on rbcL sequences. *Annals of the Missouri Botanical Garden*, **79**, 333–345.

Eriksson T (1998) *AutoDecay*, Version 4.0. Department of Botany, Stockholm University, Stockholm.

Feder ME (1983) Integrating the ecology and physiology of plethodontid salamanders. *Herpetologica*, **39**, 291–310.

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, **39**, 783–791.

Hasegawa M, Kishino H, Yano T-A (1985) Dating of the Human-Ape splitting by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution*, **22**, 160–174.

Hewitt G (2000) The genetic legacy of the Quaternary ice ages. *Nature*, **405**, 907–913.

Highton R (1995) Speciation in eastern North American salamanders of the genus *Plethodon*. *Annual Review of Ecology and Systematics*, **26**, 579–600.

Highton R, Brame AH Jr (1965) *Plethodon stormi* species nov. Amphibia: Urodela: Plethodontidae. *Pilot Register of Zoology*, Card no. 20.

Highton R, Larson A (1979) The genetic relationships of the salamanders of the genus *Plethodon*. *Systematic Zoology*, **28**, 579–599.

Jockusch EL, Wake DB (2002) Falling apart and merging: diversification of slender salamanders (Plethodontidae: *Batrachoseps*) in the American West. *Biological Journal of the Linnaean Society*, **76**, 361–391.

Kocher TD, Thomas WK, Meyer A *et al*. (1989) Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences*, **86**, 6196–6200.

Macey JR, Strasburg JL, Brisson JA *et al*. (2001) Molecular phylogenetics of the Western North American frogs of the *Rana boylii* species group. *Molecular Phylogenetics and Evolution*, **19**, 131–143.

Mahoney MJ (2001) Molecular systematics of *Plethodon* and *Aneides* (Caudata: Plethodontidae: Plethodontini): phylogenetic analysis of an old and rapid radiation. *Molecular Phylogenetics and Evolution*, **18**, 174–188.

Matocq MD (2002) Phylogeographical structure and regional history of the dusky-footed woodrat, *Neotoma fuscipes*. *Molecular Ecology*, **11**, 229–242.

Moritz C (1994a) Defining 'Evolutionarily Significant Units' for conservation. *Trends in Ecology and Evolution*, **9**, 373–375.

Moritz C (1994b) Applications of mitochondrial DNA analysis in conservation: a critical review. *Molecular Ecology*, **3**, 401–411.

Moritz C, Schneider CJ, Wake DB (1992) Evolutionary relationships within the *Ensatina eschscholtzii* complex confirm the ring species interpretation. *Systematic Biology*, **41**, 273–291.

Nielson M, Lohman K, Sullivan J (2001) Phylogeography of the tailed frog (*Ascaphus truei*): implications for the biogeography of the Pacific Northwest. *Evolution*, **55**, 147–160.

Nussbaum RA, Brodie ED Jr, Storm RM (1983) *Amphibians and Reptiles of the Pacific Northwest*. University of Idaho Press, Moscow, ID.

Palumbi SR (1996) Nucleic acids II: the polymerase chain reaction. In: *Molecular Systematics*, 2nd edn (eds Hillis DM, Moritz C, Mable BK), pp. 205–247. Sinauer Associates, Sunderland, MA.

Petranka JW (1998) *Salamanders of the United States and Canada*. Smithsonian Institution Press, Washington, D.C.

Posada D, Crandall KA (1998) Modeltest: testing the model of DNA substitution. *Bioinformatics*, **14**, 817–818.

Posada D, Crandall KA (2001) Selecting the best-fit model of nucleotide substitution. *Systematic Biology*, **50**, 580–601.

Riddle BR (1996) The molecular phylogeographic bridge between deep and shallow history in continental biotas. *Trends in Ecology and Evolution*, **11**, 207–211.

Roe BA, Ma D-P, Wilson RK, Wong JF-H (1985) The complete nucleotide sequence of the *Xenopus laevis* mitochondrial genome. *Journal of Biological Chemistry*, **260**, 9759–9774.

Rogers AR, Harpending H (1992) Population growth makes waves in the distribution of pairwise genetic distances. *Molecular Biology and Evolution*, **9**, 552–569.

Schneider S, Excoffier L (1999) Estimation of past demographic parameters from the distribution of pairwise differences when mutation rates vary among sites: application to human mitochondrial DNA. *Genetics*, **152**, 1079–1089.

Schneider S, Roessli D, Excoffier L (2000) *Arlequin, Version 2.000: A Software for Population Genetics Data Analysis*. Genetics and Biometry Laboratory, University of Geneva, Geneva Switzerland.

Slatkin M, Hudson RR (1991) Pairwise comparisons of mitochondrial DNA sequences in stable and exponentially growing populations. *Genetics*, **129**, 555–562.

Smith JP Jr, Sawyer JO Jr (1988) Endemic vascular plants of northwestern California and southwestern Oregon. *Madroño*, **35**, 54–69.

Soltis DE, Gitzendanner MA, Strenge DD, Soltis PS (1997) Chloroplast DNA intraspecific phylogeography of plants from the Pacific Northwest of North America. *Plant Systematics and Evolution*, **206**, 353–373.

Stebbins RC (1951) *Amphibians of Western North America*. University of California Press, Berkeley, CA.

Stebbins RC (2003) *Western Reptiles and Amphibians*, 3rd edn. Houghton Mifflin Co., Boston, MA.

Swofford DL (2002) PAUP*. *Phylogenetic Analysis Using Parsimony (*And Other Methods)*, Version 4. Sinauer Associates, Sunderland, MA.

Swofford DL, Olsen GJ, Waddell PJ, Hillis DM (1996) Phylogenetic inference. In: *Molecular Systematics*, 2nd edn (eds Hillis DM, Moritz C, Mable BK), pp. 407–514. Sinauer Associates, Sunderland, MA.

Tajima F (1989a) The effect of change in population size on DNA polymorphism. *Genetics*, **123**, 597–601.

Tajima F (1989b) Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics*, **123**, 585–595.

Tajima F (1993) Measurement of DNA polymorphism. In: *Mechanisms of Molecular Evolution: Introduction to Molecular Paleopopulation Biology* (eds Takahata N, Clark AG), pp. 37–59. Japan Scientific Societies Press and Sinauer Associates, Tokyo and Sunderland, MA.

Tan A-M, Wake DB (1995) MtDNA phylogeography of the California newt, *Taricha torosa* (Caudata, Salamandridae). *Molecular Phylogenetics and Evolution*, **4**, 383–394.

Van Denburgh J (1916) Four species of salamanders new to the state of California, with a description of *Plethodon elongatus*, a new species, and notes on other salamanders. *Proceedings of the California Academy of Sciences*, **6**, 215–221.

Vitt LJ, Caldwell JP, Zani PA, Titus TA (1997) The role of habitat shift in the evolution of lizard morphology: evidence from tropical *Tropidurus*. *Proceedings of the National Academy of Sciences USA*, **94**, 3828–3832.

Welsh HH Jr (1990) Relictual amphibians and old-growth forests. *Conservation Biology*, **4**, 309–319.

Welsh HH Jr, Lind AJ (1995) Habitat correlates of the Del Norte salamander, *Plethodon elongatus* (Caudata: Plethodontidae), in northwestern California. *Journal of Herpetology*, **29**, 198–210.

Whittaker RH (1961) Vegetation history of the Pacific Coast states and the 'central' significance of the Klamath Region. *Madroño*, **16**, 5–23.

Wiens JJ, Reeder TW (1995) Combining data sets with different numbers of taxa for phylogenetic analysis. *Systematic Biology*, **44**, 548–558.

Wilkinson M (1995) Coping with abundant missing entries in phylogenetic inference using parsimony. *Systematic Biology*, **44**, 501–514.

Meredith J. Mahoney's research focuses on the evolutionary biology of amphibians and reptiles, particularly plethodontid salamanders. She uses molecular and morphological analyses to study phylogenetic relationships across all levels from populations to species, genera, and other deeply diverged lineages.
