Genetic Diversity of Long-toed Salamanders *(Ambystoma macrodactylum)* in High-Elevation Lakes

Final Report

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ABSTRACT

The genetic population structure of long-toed salamanders (*Ambystoma macrodactylum*) from North Cascade Park was examined to investigate patterns of post-glacial colonization, population structure, gene flow, and genetic impacts from predation by introduced trout. Eight polymorphic microsatellite loci revealed surprising genetic diversity both within and among local populations. Genetic and geographic distances between populations were positively correlated, and estimated migration levels between populations were very low. These indicate genetic isolation of populations increases with geographic distance, and colonization may be a gradual process. Genetic diversity of salamander populations sympatric with non-reproducing trout was not significantly lower than genetic diversity of populations living without trout. Patterns of genetic diversity within the studied populations revealed two significantly distinct genetic groups representing higher elevation, east slope (Stehekin drainage) populations and lower elevation, west slope (Skagit drainage) populations. This divergence may represent colonization of NOCA's high-elevation lakes from separate glacial refugia.

INTRODUCTION

Concern about the decline of amphibian species is particularly acute in areas such as high elevation locations in the western US that have been relatively undisturbed by human activities (Blaustein and Wake 1990). Fish are not indigenous to most high-elevation lakes in the west (Bahls 1992), and introduction of fishes including trout has been implicated as a possible cause of amphibian declines (e.g., Taylor 1983; Bradford 1989; Bradford et al. 1993; Fellers and Drost 1993; Blaustein et al. 1994; Tyler et al. 1998a).

To persist in high-elevation environments, species must maintain genotypes that are adapted to harsh and variable environmental conditions. The genetic diversity of a population provides its capacity to adapt to changes in the environment and it is a critical parameter in assessing the risks of human activities to indigenous populations (Reh and Seitz 1990; Olsen et al. 1996). In general, genetic diversity increases with increased population size (Hartl and Clark 1997a) so that the largest populations will have the greatest level of diversity.

Trout predation could reduce genetic diversity of salamanders both by reducing salamander population density and by selectively removing the phenotypes that behaviorally are the most vulnerable to predation (Storfer and Sih 1998). In addition, extirpation of populations could increase isolation among populations and reduce dispersal and gene flow. Reduced dispersal has been associated with increased probability of metapopulation extinction (Hanski 1991; Sjogren 1991; Sjogren-Gulve and Ray 1996). Of particular concern are the impacts of fish on large salamander populations, which may be analogous to core populations (*sensu* Harrison 1991, 1994) in a metapopulation. Core populations serve as repositories for genetic diversity and relatively stable sources of dispersers that can recolonize adjacent habitats where local population extinctions have occurred.

Since 1989, we have been conducting research on the impacts of introduced trout on native biota, including long-toed salamanders (*Ambystoma macrodactylum),* in North Cascades National Park Service Complex (NOCA). Long-toed salamanders are the top carnivores in high-elevation fishless NOCA lakes (Tyler et al. 1998a). Though naturally fishless, many lakes have been stocked with cutthroat (*Oncorhynchus clarkii*) and rainbow trout (*Oncorhynchus mykiss mykiss*) to provide recreational opportunities. In some NOCA lakes, trout have established reproducing populations that often reach high densities (405-650 fish/ha; Gresswell et al. 1997), but in lakes where trout cannot reproduce, juvenile fish (fry) are periodically stocked (average interval between stocking >5 years) at low densities (average 179 fish/ha; Liss et al. 2002). Many anglers prefer to fish in lakes with non-reproducing trout because trout densities are low and individual fish reach a large size. Because trout can be eliminated in a few years simply through cessation of stocking, lakes with non-reproducing trout may offer the most options for future management for lake restoration if deleterious effects on native biota are demonstrated. In contrast, if fish impacts on native biota are not evident in non-reproducing fish lakes, NOCA could consider allowing stocking of these lakes to continue.

Our research has shown that, in fishless lakes, there was a significant positive relationship between total Kjeldahl nitrogen (TKN), an indicator of lake productivity (Lambou et al. 1983; Paleheimo and Fulthorpe 1987), and larval salamander density (Tyler et al. 1998a). Increased larval density was related to increased availability of cladocerans, an important food resource for larval salamanders. The effect of trout on larval salamander density appears to depend primarily on the concentration of TKN in a given lake. In lakes with low TKN ($< \sim 0.05$ mg/L), there was no significant difference in larval densities between fishless lakes and lakes with trout (Tyler et al. 1998a, Liss et al. 2002), as salamander densities were very low regardless

of whether trout where present or absent. In contrast, at high TKN (> ~ 0.05 mg/L), conditions where salamander larvae likely would have been abundant, larval abundance was significantly higher in fishless lakes than in lakes with fish (Tyler et al. 1998a), including lakes with non-reproducing trout (Liss et al. 2002). Larval salamanders were absent in nearly all lakes with reproducing trout within this TKN range.

The principle goal of this project was to test the hypothesis that introduced trout have had no significant effect on within-and among-population genetic diversity of high-elevation (>800 meters) *A. macrodactylum* populations in NOCA. Four primary objectives were involved:

1. Determine the relationship between within-population genetic diversity of salamanders and larval salamander population size.
2. Determine within-population genetic diversity of larval salamanders in lakes with non-reproducing trout and compare it to genetic diversity in fishless lakes.
3. Determine genetic relationships among salamander populations in fishless lakes and lakes with non-reproducing fish.
4. Determine genetic relationships among populations within a watershed (the Skagit and Stehekin drainages) and between watersheds.

Accurate assessment of these genetic parameters within and among populations on the fine scale of local populations in NOCA requires the study of genetic markers that have a bi-parental mode of inheritance. Previous studies have examined allozymes, which require lethal sampling, to document genetic variation and gene flow in Ambystomatid salamanders (Howard and Wallace 1981; Jones 1989; Titus 1990; Shaffer et al. 1991; Larson and Dimmick 1993; Storfer and Sih 1998, Tallmon et al. 2000). This would be unacceptable for studies of *A. macrodactylum* in NOCA, since the removal of ten to twenty individuals for genetic studies may translate into

the loss of a significant proportion of the animals in many of the small, local populations. Genetic investigations for this study utilized the polymerase chain reaction (PCR) (Avise 1994; Bruford and Wayne 1993; Burke et. al. 1991) to amplify large quantities of specific targeted DNA sequences from minute amounts of tissue, eliminating the need for lethal sampling.

METHODS

Tissue Collection and Estimation of Population Abundance

Two species of Ambystomatid salamanders (*A. macrodactylum* and *A. gracile*) occur in NOCA, but they were not observed together in any of the study lakes. In NOCA, *A. gracile* is generally restricted to lower-elevation lakes (Liss et al 1995b), and most collections of larval tissue for this study were made in higher-elevation lakes where *A. gracile* rarely occurs

Roughskinned newts (*Taricha granulosa*) were observed in one of the study lakes (RD3), so care was taken in field identification at the time of tissue excision. Larvae in sampled lakes were determined to be *A. macrodactylum* based on larval characteristics (Leonard et al. 1993; Corkran and Thoms 1996), absence of large larvae (>60mm total length) or neotenes and egg masses characteristic of *A. gracile*, and the presence of premetamorphic individuals with adult coloration.

A hand-held sonar gun was used to determine maximum depth of each lake. Lake elevations were derived from 7.5 min USGS topographical maps, and lake surface areas were determined by digitization of lake shorelines outlined on these maps (Table 1). During each sampling visit, water samples were collected from one meter below the lake surface with a 1.5 L van Dorn-style sampling bottle. Water samples were gathered over each lake's deepest point to standardize sampling between lakes. One liter of collected water was filtered in the field using a

Whatman GF/C mesh glass fiber filter, and water samples were frozen immediately upon return from the field prior to analysis. Frozen filtered and unfiltered water samples were analyzed for total Kjeldahl-N (Table 1) and other chemical parameters at the Cooperative Chemical Analytical Laboratory at Oregon State University, Corvallis, Oregon.

Table 1. Physical variables of eight NOCA lakes from which salamander larvae were sampled

Lake	Elevation, (m)	Maximum Depth, (m)	Surface Area, (ha)	TKN, (mg/L)
DD1	1496	2.4	0.5	0.09
LS3FS	1375	6.7	1.2	0.02
MC7	1556	27.2	6.7	0.02
MR12-93,98	1981	4.0	0.6	0.125, 0.06
MR13-1	1800	2.0	0.3	0.06
MR2	1873	1.5	0.3	0.09
MR3-93,99	1873	2.5	0.2	0.14, 0.06
RD3-94,99	802	8.8	0.3	0.08, 0.07

We determined the density of larval salamanders and processed tissue samples for genetic analysis from eight lakes in NOCA sampled between 1993 and 1999 (Table 2). To assess temporal changes in genetic diversity within populations, tissue samples were processed from archived and recent collections from lakes RD3 (1994, 99), MR3 (1993, 99) and MR12 (1993, 98). Although MR 12 was fishless in 1993, fish were present in the lake in 1998.

It is very difficult to assess accurately the abundance of adult *A. macrodactylum* in high-elevation areas in NOCA, so it was assumed that larval abundance was related to adult population sizes. Larval salamander density was estimated by snorkel surveys (Tyler et al. 1998a). Because of the remoteness of lakes (they could be reached only by hiking or helicopter), snorkeling provided the best estimates of larval densities given constraints of time and equipment. Four, 25-meter segments of shoreline were chosen randomly along the perimeter of

Table 2. Summary of data on NOCA long-toed salamander populations.

Lake	East/ West	Fish +/-	Sample Date	Population Density Estimate (Number/100m)	Lake Perimeter (m)	Estimated Total Larval Population	Number Individuals Used for Genetic Analysis	% Population Used For Genetic Analysis
DD1	W	+	Jul-98	2	280	5.6	5	89%
LS3FS	W	+	Sep-99	4	479	19.16	4	21%
MC7	W	+	Sep-99	11	992	109.12	6	5%
MR 12-93	E	-	Jul-93	110	284	312.4	5	2%
MR12-98	E	+	Aug-98	6	284	17.04	4	23%
MR13-1	E	-	Aug-93	20.3	194	39.38	4	10%
MR2	E	-	Aug-99	65	214	139.1	20	14%
MR3-93	E	-	Aug-93	38.7	144	55.73	3	5%
MR3-99	E	-	Aug-99	14	144	20.16	20	99%
RD3-94	W	-	Jun-94	81	146	118.26	7	6%
RD3-99	W	-	Aug-99	41	146	59.86	18	30%
Average				35.7	300.6	81.4	8.7	28%

each lake (all study lakes were < 7 ha in area, and all but two were less than one ha). During mid-afternoon, a snorkeler carefully searched through substrate material (talus, woody debris, fine organic material, and aquatic vegetation) within two meters of the shoreline and recorded the number of larvae observed. We found no statistically significant difference in larval *A. macrodactylum* density between surveys conducted during the day and at night (Tyler et al. 1998a).

We extrapolated total larval population sizes (n) from estimates of salamander densities using the following equation:

n = d x p, where

d = density of salamanders (number of larvae per 100 linear meters of sampled shoreline) and

p = perimeter of lake (meters).

The presence of trout in lakes was determined from stocking records and confirmed by gill netting, angling, and snorkeling.

Small quantities of tail tissue from larval salamanders were collected concurrently with estimation of population density. Tissue was excised from non-vascularized caudal finfold areas, without incursion into muscular tissue. Minimal or no bleeding was observed in all specimens, and no mortalities were documented. All tissue samples were preserved in either 70% ethanol or Modified Queen's Buffer (10mM Tris pH 7.5, 100mM EDTA pH 8, 5% v/v DMSO, saturated with NaCl) and stored at ambient temperature in screw-top 1.5 ml polypropylene tubes with silicone gaskets. Though sample sizes were small, they often represented a significant proportion of the estimated total number of larvae present in the population in each lake at the time of sampling (range 2-99% of the estimated larval population; Table 2).

Tissue samples were also collected from specimens of *A. macrodactylum* from the Willamette Valley in Benton County, Oregon. These larvae were collected from a small (12 m^2) temporary pond adjacent to railroad tracks near Finley Wildlife Refuge. These samples were meant to serve as an 'outgroup,' crucial for 'rooting' phylogenetic trees and interpreting the significance of among-population variation documented in nuclear genes among salamander populations within NOCA. Extensive attempts were made during two field seasons to collect tail tissue from adult *A. macrodactylum* from populations below 800 m in the Skagit and Stehekin drainages, but with no success.

Genetic Analysis

We explored a variety of methodologies to generate genetic data before we found one that would provide resolution adequate for accomplishing the objectives of this research. Hypervariable spacer regions of mtDNA were amplified and sequenced as described in McKnight and Shaffer (1997) from a subsample of one individual from each of the study lakes, plus an individual from the Willamette Valley outgroup population. However, no useful diversity was documented among the mitochondrial sequences examined from NOCA samples, so this analysis was not pursued further.

Generic microsatellite probes (Jeffries probes, etc.) did not provide polymorphisms that were useful for resolving the population-level questions we needed to address for this study, and Southern blot probing required too much DNA for the quantity of tissue we could collect with non-lethal sampling methods. Primers developed for PCR amplification of hypervariable single-copy nuclear genes and microsatellites from other amphibian species did not work for *A. macrodactylum*, despite attempts to optimize conditions for annealing and amplification. This

included micro- and minisatellites from the toad *Bufo bufo* (Scribner et. al. 1994) and microsatellite primers developed for newts (*Taricha*). Though the latter yielded no amplification products in *A. macrodactylum*, they may prove useful for screening future tissue samples for *Taricha* larvae that may be included incidentally during field collection.

It became apparent that the only way to uncover levels of genetic variability useful for our study was to develop microsatellite primers specific for this species. This was achieved by contracting a private biotechnology firm, Genetic Identification Services (GIS). GIS produced and screened a DNA library for microsatellite primers for *A. macrodactylum*, optimized the amplification conditions, and performed and scored PCR amplifications of all twelve microsatellite loci. High confidence can be placed in the results generated in this laboratory, as they are certified for undertaking forensic DNA contracts with various law-enforcement agencies.

Construction and screening of a DNA library for tri- and tetra-nucleotide microsatellite motifs yielded twelve loci that were highly polymorphic and reproducible among NOCA and/or the outgroup population of *A. macrodactylum*. Specific forward and reverse primer pairs were designed for each locus (Appendix 1) and amplification conditions were optimized to minimize 'stutter' and maximize band intensity.

The possibility of 'null alleles' was investigated at each locus in each population where homozygous overabundance or heterozygous deficiency was found. The phenomenon of 'null alleles' indicates that PCR amplification of some alleles failed because mutations have occurred in the DNA at or near primer binding sites of those alleles. The presence of 'null alleles' can create the artifact of apparent homozygote overabundance or heterozygous deficiency, since the single allele of a successfully amplified product is indistinguishable from (and will be interpreted

as) a homozygous condition. This can mimic homozygosity in truly heterozygous individuals, and in analysis of populations creates an artifact that may be indistinguishable from depressed genetic diversity (resulting from bottlenecks, inbreeding or small population sizes) or the Wahlund Effect (absence of heterozygotes due to the inclusion of genetically distinct, reproductively isolated demes within a single sample, Tallmon et al. 2000). Primer refinement and testing enabled us to discern that the apparent homozygosity in NOCA populations was a real indication of a loss of genetic diversity at two loci (F104, F12b), but likely an artifact of 'null allele' amplification for four other loci (F136, F11, H18, H120; Table 3). These four problematic loci were dropped from subsequent analyses for this study. Analyses and conclusions are based on eight polymorphic microsatellite loci that amplified successfully in all populations.

Data Analysis

Data derived from microsatellite results were analyzed with the computer programs Genepop (version 1.2, Raymond and Rousset 1995), Genetix (version 3.3, Belkhir et al. 2001) and Phylip (version 3.6, Felsenstein 2001). Tests were performed for Hardy-Weinberg equilibrium in each local population using chi-square goodness-of-fit-tests. All populations were assessed for linkage disequilibrium across all loci using 50 iterations for each allele of each of the eight study loci in each population as well as for each allele in a pooled sample including all populations. Estimates of within-population diversity were generated, both as mean heterozygosity (*sensu* Stephens et. al. 1992) and as the average number of alleles per locus. Genetic distance between each pair of populations was estimated using the DIST operation of Genepop, and as pairwise genetic distance (Nei 1972, 1978) and 'D' of Reynolds et al. (1983)

Table 3. Number of alleles and their sizes (in base pair length of amplified product) detected in a survey of twelve microsatellite loci surveyed among 110 *Ambystoma macrodactylum* from NOCA, Northern Washington and eight from the mid-Willamette Valley, Oregon. Asterisk(*) denotes alleles documented only in the outgroup population; @ indicates alleles found in NOCA populations but trimmed from final data set.

Locus	**F136**	**F11**	**F104**	**H136**	**H129**	**H18**	**H20**	**H29**	**F142**	**H120**	**F12b**	**H123b**
Total # alleles documented	**12**	**15**	**5**	**13**	**14**	**8**	**16**	**8**	**12**	**19**	**5**	**12**
# alleles documented in NOCA populations	**4**	**10**	**3**	**11**	**13**	**2**	**14**	**4**	**9**	**9**	**4**	**8**
Alleles used in analysis	**0**	**0**	**3**	**11**	**13**	**0**	**12**	**4**	**9**	**0**	**3**	**8**
	159	156	196*	127*	202	238*	236@	191	160	282*	170@	233*
	168	163	202	131	206	243	246	195	163*	286*	173	241
	174	168	208	136	210*	251*	250	199	166	288	176*	245
	177*	172	211*	140	214	255*	254	204	168	291	179	249
	180*	175*	214	144	218	259*	260@	208*	169	294	182	253
	183*	178		156	222	263	262*	212*	172	298*		257
	186*	182		160	226	275*	268	220*	176	300*		261
	189*	186		164*	230	279*	270	232*	179*	306*		265
	192*	189		168	234		272		181	314*		269*
	195*	192*		172	238		274		184	318*		273
	201*	195*		176	242		278		185	322*		277*
	215	198		180	246		282		190*	328*		281*
		205*		184	250		286			330		
		208*			254		290			353*		
		214					308			395		
							318*			399		
										402		
										403		
										407		

with Genetix, utilizing 'theta' as the Fst estimator. Although absolute values were different, general trends in relative values between populations were similar for all methods of distance calculation. Nei's (1978) estimator of genetic distance among population pairs was utilized for subsequent statistical analyses. Two dimensional graphs of principal components scores of allelic diversity of all individuals were produced with scores generated from Genetix.

The fixation index, Fst of Wright (1978), is an estimate that compares the average heterozygosity of a local population to the level of average heterozygosity observed in the collective metapopulation. Fst is an all-inclusive measure of population substructure and a sensitive indicator of how genetically unique a population is relative to all others included in the study (Hartl and Clark 1997b). We estimated Fst values for each allele at each of eight loci among all populations in the study as 'theta' using the methods of Weir and Cockerham (1984). In addition to pairwise comparisons with single loci, we estimated the average Fst for multiple alleles as a weighted average of Fst values for all loci examined (Hartl and Clark 1997b) across all populations as a quantitative estimate of how divergent each population was from other populations in the study.

Estimates of gene flow (Nm, number of migrants per generation) were calculated utilizing Wright's 1969 equation (1-Fst) / (4 * Fst), with 'theta' of Weir and Cockerham (1984) as the Fst estimator. Values were derived using the operation GSED (Lewis and Zaykin 1996) in Genetix.

Linear geographic distance between each pair of populations was determined from GPS coordinates analyzed with the GIS program Arcview. Simple linear regression was used to determine relationships between genetic distance, Nm and lineal geographic distance between all lake pairs. Statistical analyses were conducted using Statgraphics Plus (Version 5). In addition, significance of correlations between genetic and geographic distance were tested with the

MANTEL operation of Genepop.

Nei's genetic distance was also utilized by the program Gendist operation in Phylip to generate phylogenetic trees. Fifty iterations included randomized order of populations and subsamples of n = 4 individuals (smallest sample size) from each population. Heuristic searches were used to find the shortest possible branch lengths using the neighbor-joining method, and negative branch lengths were allowed. Bootstrapping was performed on the twenty most parsimonious trees; nodes of the consensus tree presented were supported more than 50% of the time.

Average number of alleles per locus and average heterozygosity were compared between lakes with and without fish using the Mann-Whitney Test. Stepwise multiple regressions were employed to examine relationships between number of alleles per locus and average heterozygosity, which were the response variables, and estimated salamander population abundance, number of larval tissue samples analyzed from each population (sample size), aspect (whether a lake was located on the east or west slope of the Cascades), presence or absence of fish, lake elevation (m), maximum depth (m), and surface area (ha). Lake aspect and fish presence were entered into the models as categorical variables. Stepwise selection of variables was terminated when variables added to models were no longer significant at $\alpha \leq 0.05$.

RESULTS

Principal components analysis revealed that the Willamette Valley outgroup population was genetically distinct from the NOCA populations (Figure 1).

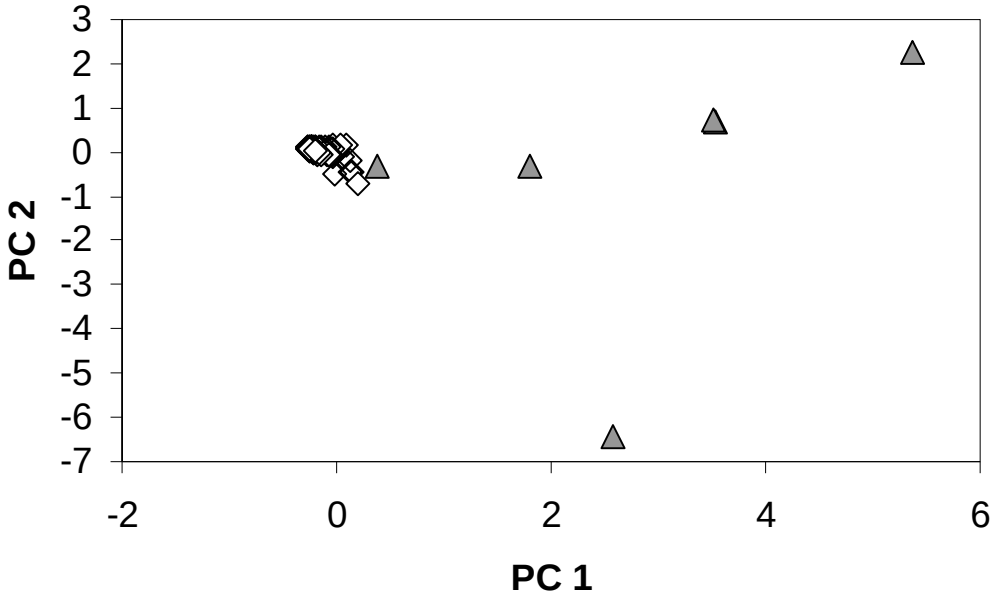

Figure 1. First and second principal components of genotypes for eight microsatellite loci for NOCA (open diamonds) and Willamette Valley outgroup (closed triangles) populations of long-toed salamanders

The first principal component accounted for 10.97 % of the variation in allelic diversity, and the second principal component accounted for 7.64 % of the variation in this analysis. The remote Willamette Valley outgroup was so different from all NOCA populations that its inclusion in principal components analyses obscured the real yet subtle genetic variation among local NOCA populations.

Principal components analysis was repeated on a data set excluding the outgroup population in order to reveal interpopulational relationships within NOCA crucial for resolving the questions proposed in this study. In this analysis, the first principal component accounted for 9.18 % of the variation in allelic diversity, and the second principle component accounted for 6.15 % of the variation Figure 2). This second principal components analysis revealed segregation of salamander populations occurring on the east and west slope of the

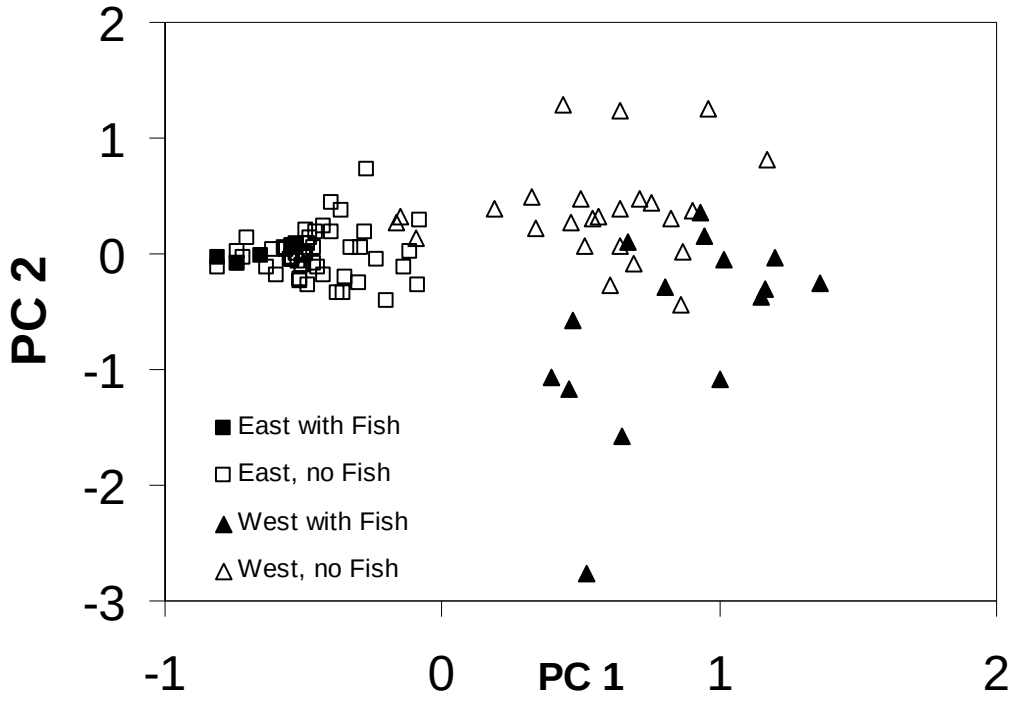

Figure 2. First and second principal components of genotypes of eight microsatellite loci from east slope (Stehekin Drainage) and West Slope (Skagit Drainage) NOCA salamander populations with and without fish (Willamette Valley outgroup excluded).

Cascade Mountains. East slope (Stehekin Drainage) populations were more constrained in multivariate space suggesting lower levels of genetic diversity than west slope (Skagit) populations. In addition, there was no evidence of selective genetic changes in the genetic structure of populations sympatric with trout, as principal component scores generated for individuals from those populations generally fell within the ranges of animals from other populations within the same drainage basin (Stehekin vs. Skagit; Figure 2).

A total of twelve highly polymorphic (at least 5 alleles per locus) microsatellite loci were identified (Table 3, Appendix 1), though not all alleles were found in the NOCA populations. From three to thirteen alleles were documented at each of eight loci among all NOCA salamanders examined (F136, F11, H18, H120 excluded; Table 3). The average number of

alleles per locus ranged from 1.5 to 5.125 (Table 4). Allelic diversity was generally higher in west-slope populations than in east-slope populations (Table 4), consistent with the results of principal components analysis. All loci showed "moderate" to "very great" levels of genetic differentiation among populations as assessed by Fst values (Table 5). Loci H136, H129, H20, and H123b were highly polymorphic in some of the study populations (Table 4).

There was no evidence of linkage disequilibrium among any loci, so there was no apparent violation of independent assortment of loci analyzed in this study. There was no evidence of the Wahlund Effect in any of our sampled populations, indicating larvae collected from each lake at any given time represented the progeny of a single breeding population without reproductive subdivision within lakes at any one sampling date.

Stepwise regression analysis indicated that the average number of alleles per locus for each population was positively related to sample size and negatively related to lake elevation ($p < 0.0001$; $R^2 = 0.96$, $n = 10$; Figures 3 and 4). The model was:

$$\text{Alleles per locus} = 4.65 + \beta_1 \text{ Sample size} - \beta_2 \text{ Elevation}$$

(where $\beta_1 = 0.12$ (+/- 0.02) and $\beta_2 = 0.002$(+/- 0.0002).

Table 4. Average and individual number of alleles documented in each population for each of eight microsatellite loci from *A. macrodactylum* populations in NOCA.

Population	Average # alleles per locus	Average Heterozygosity	F104	H136	H129	H20	H29	F142	F12b	H123b
DD1	2.75	0.40	2	4	4	2	2	3	2	3
LS3FS	2.75	0.53	1	4	3	5	1	2	2	4
MC7	3.25	0.56	1	5	4	4	3	3	2	4
MR12-93	1.75	0.25	1	2	2	3	1	3	1	1
MR12-98	1.5	0.125	1	2	2	2	1	2	1	1
MR13a	2.25	0.34	2	3	3	3	1	2	1	3
MR3-93,99	4.25	0.39	2	9	5	7	1	4	2	4
MR2	4.00	0.42	2	9	5	6	1	3	2	4
RD3-94	5.125	0.61	2	5	10	6	4	5	2	7
RD3-99	4.25	0.54	3	5	6	6	3	4	2	5
Average	3.19	0.42	1.7	4.8	4.4	4.4	1.8	3.1	1.7	3.6
East	2.75	0.31	1.6	5.0	3.4	4.2	1.0	2.8	1.4	2.6
West	3.63	0.53	1.8	4.6	5.4	4.6	2.6	3.4	2.0	4.6
% greater in West	31.8	73.11	12.5	-8.0	58.8	9.5	160.0	21.4	42.9	76.9

Table 5. Degree of genetic differentiation of alleles at each locus among NOCA populations of *A. macrodactylum* using the guidelines of Wright (1978) for the significance of Fst values: little (0-0.05), moderate (.05-0.15), great (0.15-0.25), and very great (above 0.25).

		Degree of Genetic Differentiation Among Populations		
Locus	Average Fst	Moderate	Great	Very Great
F104	0.475			X
H136	0.142	X		
H129	0.300			X
F142	0.270			X
F12b	0.113	X		
H29	0.172		X	
H20	0.194		X	
H123	0.234		X	
Multilocus	0.221		X	

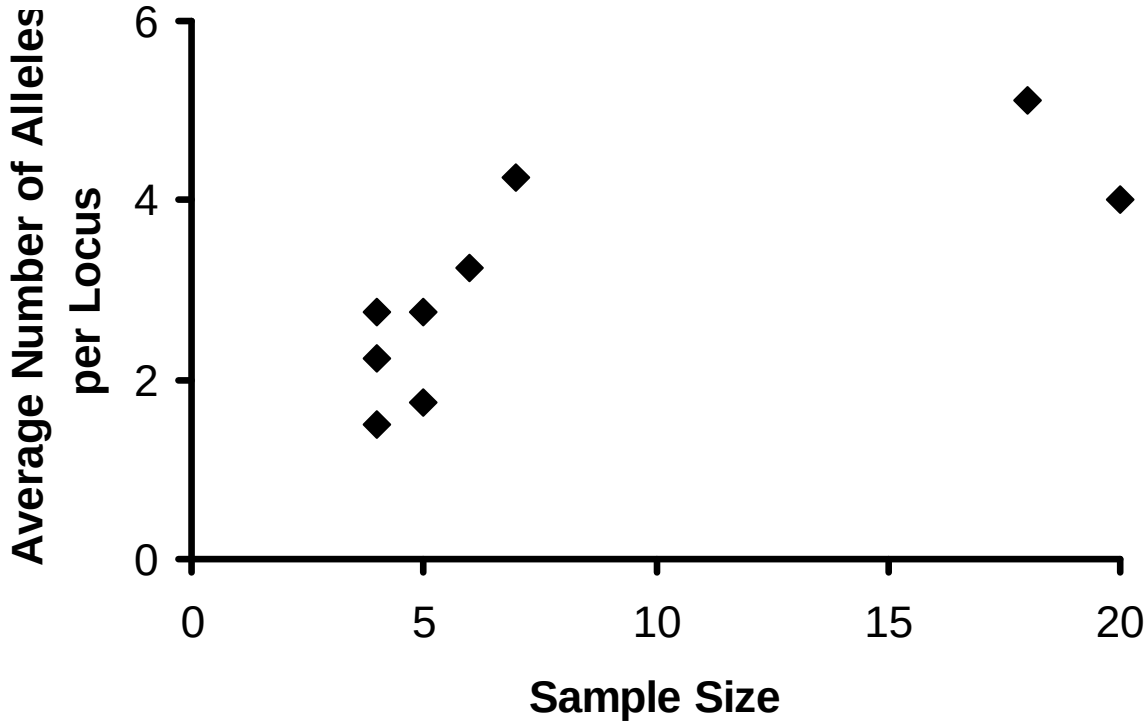

Figure 3. Relationship between the average number of alleles per locus detected in each salamander population and sample size.

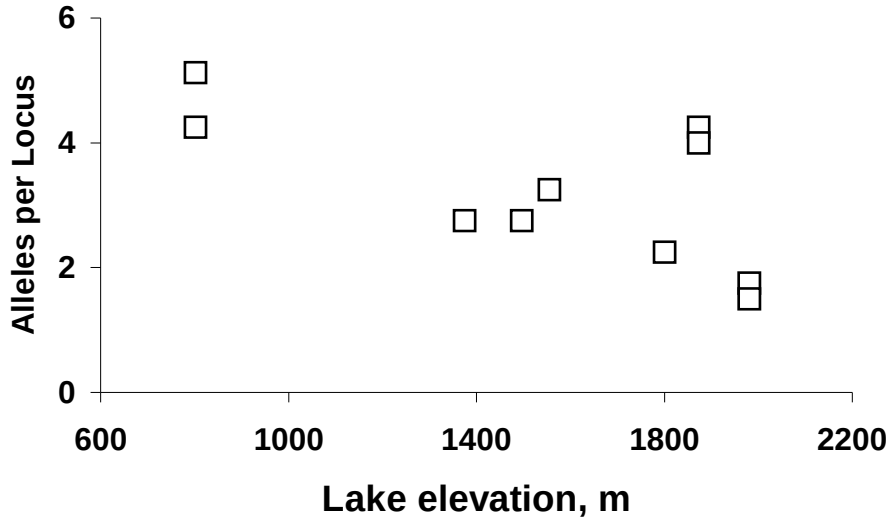

Figure 4. Relationship between alleles per locus and lake elevation of NOCA *A. macrodactylum* populations.

In the regression model, a slightly greater proportion of the total variation in larval alleles per locus was explained by sample size (partial $R^2 = 0.93$) than by elevation (partial $R^2 = 0.88$).

Another significant model related alleles per locus to sample size and lake aspect ($p = 0.0003$; $R^2 = 0.90$; $n = 10$). This model was:

$$\text{Alleles per locus} = 3.82 + \beta_1 \text{ Sample size} - \beta_2 \text{ Aspect}$$

(where $\beta_1 = 0.14$ (+/- 0.02) and $\beta_2 = 1.32$ (+/- 0.28).

A somewhat greater proportion of the total variation in larval alleles per locus was explained by sample size (partial $R^2 = 0.88$) than by aspect (partial $R^2 = 0.73$).

Elevation and aspect were highly correlated (Pearson's correlation coefficient = 0.82; $p = 0.004$). The high-elevation lakes in this analysis tended to be located on the east slope of the Cascades and had a lower number of alleles per locus than the lower elevation lakes that were

located on the west slope**.** Neither population size nor presence or absence of fish was a significant variable in either regression model. Simple linear regression also failed to a find a significant relationship between alleles per locus and population size ($p = 0.77$).

Average heterozygosity ranged from 0.125 to 0.61 (Table 4). Observed heterozygosities did not differ significantly from chi-square predictions of expected heterozygosity ($p>0.5$) at 95% level of confidence in any of the study populations. Average heterozygosity was not related to sample or population size, but was weakly related to maximum lake depth ($p = 0.0435$; $R^2 = 0.42$). The regression model was:

$$\text{Heterozygosity} = \beta_0 + \beta_1 \text{Maximum Depth}$$

(where $\beta_0 = 0.16$ (+/- 0.07) and $\beta_1 = 0.20$ (+/- 0.007)

Great (0.15-0.25) and very great (above 0.25) Fst values were found among most pairs of populations (Table 6). Analyses with Genetix, Genpop, and Phylip using both allele frequencies and genotype frequencies for each population revealed that Fst values were significantly different among populations. Similar tree topologies of interpopulational relationships were obtained with both data types.

Random sub-sampling of $n = 4$ to compare like sample sizes among populations yielded similar results. This indicates a significant degree of genetic differentiation among populations within NOCA.

Genetic distance was positively related to geographic distance between populations ($p < 0.0001$; $R^2 = 0.67$; $n = 44$; Figure 5). The model was:

$$\text{Genetic distance} = 0.07 + \beta_1 \text{ Geographic distance}$$

(where $\beta_1 = 0.009$ (+/- 0.001).

Table 6. Estimates of genetic distance [Fst as Theta of Weir and Cockerham (1984) as produced from Genetix based on eight polymorphic microsatellite loci] and geographic distance between population pairs. Numbers above diagonal are Fst values; numbers below diagonal are geographic distances in km. Values with 'zero' geographic distance indicate replicate samplings from larvae in lakes MR12 and RD3 (Pyramid).

	DD1	**LS3FS**	**MC7**	**MR12-93**	**MR12-99**	**MR13-1**	**MR2**	**MR3**	**RD3-99**	**RD3-94**
DD1	0	0.289	0.240	0.443	0.486	0.352	0.334	0.35	0.200	0.168
LS3FS	31.719	0	0.017	0.539	0.552	0.362	0.350	0.366	0.076	0.092
MC7	35.677	36.65	0	0.480	0.489	0.355	0.358	0.371	0.106	0.089
MR12-93	48.928	67.003	84.624	0	0.012	0.249	0.161	0.205	0.326	0.286
MR12-99	48.928	67.003	84.624	0	0	0.277	.218	0.265	0.334	0.29
MR13-1	47.808	64.885	83.408	2.639	2.639	0	0.144	0.182	0.195	0.130
MR2	47.987	69.317	83.622	8.622	8.622	10.94	0	0.016	0.182	0.177
MR3	47.855	69.305	83.522	7.773	7.773	10.04	0.842	0	0.2	0.198
RD3-99	5.692	31.516	40.706	43.945	43.945	42.684	43.639	43.427	0	0.016

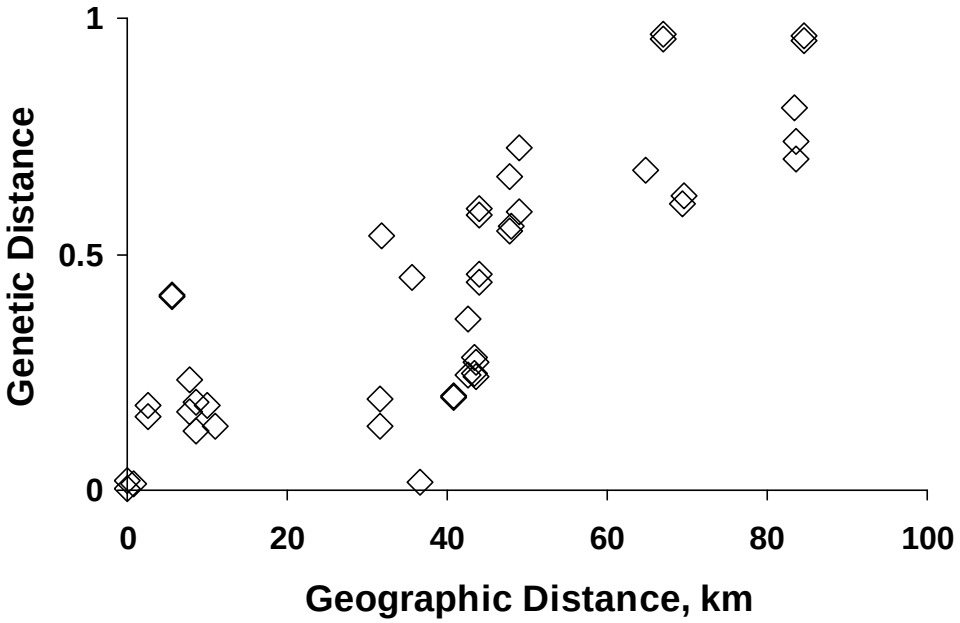

Figure 5. Relationship between genetic distance (Nei's 1978 value) and geographic distance between each pair of salamander populations in NOCA.

In addition, the rank correlation of data from NOCA populations from the MANTEL operation of Genepop also suggested isolation by distance ($P< 0.05$). The lowest values of genetic distance were estimated for replicate samples from populations in MR3, RD3, and MR12 (before and after the addition of fish; Table 6). The lowest genetic distance for geographically distinct populations was estimated from larvae in lakes MR2 and MR3, which lie in close physical proximity to one another.

There was no significant relationship between number of migrants per generation (Nm) and geographic distance ($p = 0.067$; $n = 42$; Figure 6), probably because estimates of Nm were so low overall (Table 7). Aside from the populations that were sampled twice (RD3 and MR12),

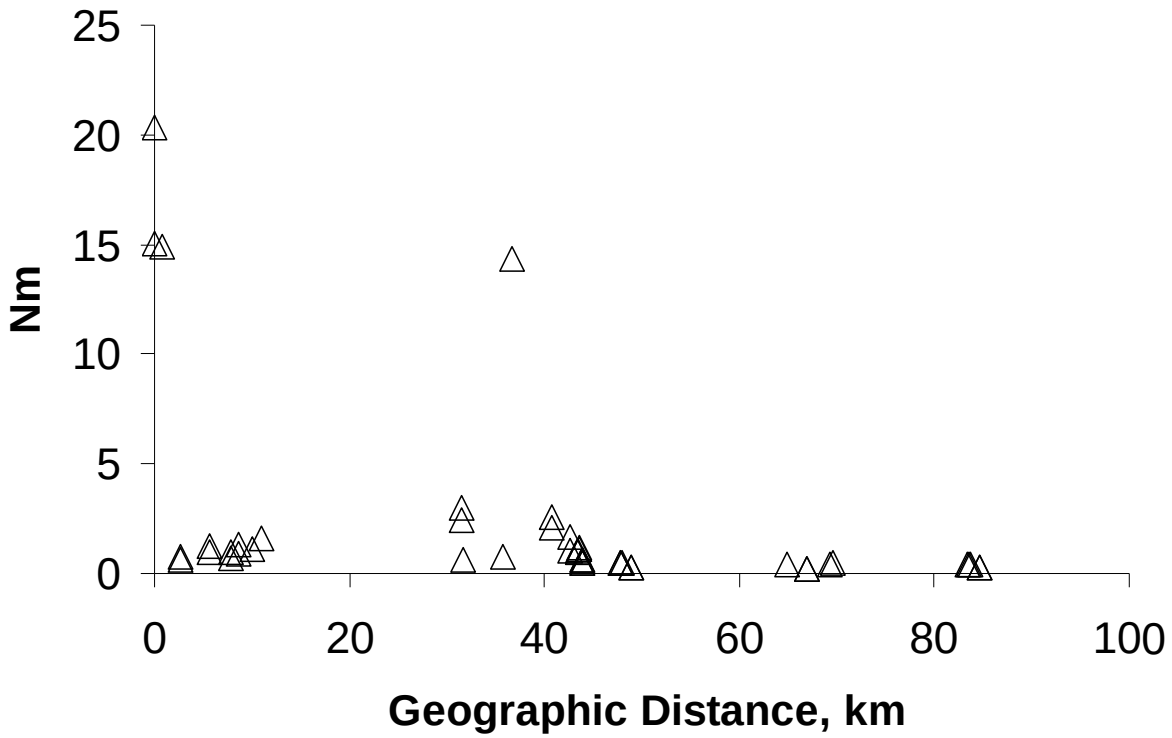

Figure 6. Relationship between the number of migrants per generation (Nm) and the geographic distance between each pair of salamander populations in NOCA.

the largest Nm was found for MR2 and MR 3, lakes in very close geographical proximity.

The salamander population of RD 3 underwent a significant decline in density due to a series of severe winters between the sampling periods; the population appeared to be at Hardy – Weinberg equilibrium at each sampling date. However, the average number of alleles per locus decreased from 5.125 to 4.25, and average heterozygosity declined from 0.61 to 0.54 between the two sampling periods (Table 4). The reduction in genetic diversity was due primarily to the decreased number of alleles documented at four loci (H129 from 10 to 6 alleles; H29 from 4 to 3 alleles; F142 from 5 to 4 alleles; H123b from 7 to 5 alleles; Table 4) at the later sampling date. This natural population decline apparently resulted in the loss of some alleles from the

Table 7. Pairwise estimates of geographic distance and rates of migration between populations. Numbers above diagonal are geographic distances in km and numbers below diagonal are Nm, number of migrants between populations per generation. Values with 'zero' geographic distance indicate replicate samplings from larvae in lakes MR12 and RD3.

Population	DD1	LS3FS	MC7	MR2	MR3	MR 12-93	MR 12-99	MR13-1	RD3-99	RD3-94
DD1	0	31.719	35.677	47.987	47.855	48.928	48.928	47.808	5.692	5.692
LS3FS	0.61	0	36.65	69.317	69.305	67.003	67.003	64.885	31.516	31.516
MC7	0.79	14.35	0	83.622	83.522	84.624	84.624	83.408	40.706	40.706
MR2	0.5	0.46	0.45	0	0.842	8.622	8.622	10.94	43.639	43.639
MR3	0.47	0.43	0.42	14.89	0	7.773	7.773	10.04	43.427	43.427
MR 12-93	0.31	0.21	0.27	1.31	0.97	0	0	2.639	43.945	43.945
MR 12-99	0.26	0.2	0.26	0.9	0.69	20.35	0	2.639	43.945	43.945
MR13-1	0.46	0.44	0.45	1.62	1.13	0.76	0.65	0	42.684	42.684
RD3-99	1	3.02	2.1	1.13	1	0.52	0.5	1.03	0	0
RD3-94	1.24	2.47	2.55	1.16	1.01	0.62	0.61	1.67	15.01	0

population. These differences were not due to a sample size effect, as more larvae were collected for the 1999 sampling period (n=18) than in the 1994 sample (n = 7).

MR12 was the only lake from which we have genetic samples of salamander larvae before and after the addition of fish. Genetic diversity in MR 12 decreased after the addition of trout. The average number of alleles per locus decreased from 1.75 to 1.5 (Table 4) due to changes in number of alleles at loci H20 and F142, where 3 alleles were detected in the first sampling and only 2 alleles in the second (Table 4). Average heterozygosity decreased from 0.25 to 0.0125 (Table 4). It is difficult, however, to directly attribute these changes to the addition of fish because similar losses in number of alleles per locus were observed between the two sampling periods in RD3, which did not support fish. In addition, sample sizes were small (four salamander larvae before addition of trout and five after the addition of trout), and the observed variation could be attributed solely to a sample size effect. There was no significant difference in the average number of alleles per locus (p=0.23, Mann-Whitney Test) and average heterozygosity (p = 0.91, Mann-Whitney Test) between lakes with fish and those without fish.

The most frequently-supported dendrogram of relationships among NOCA populations of *A. macrodactylum* is presented in Figure 7. Although details of branch arrangement varied, all dendrograms consistently separated East and West populations into separate clades. Even in runs where the Willamette Valley population was not designated as an outgroup for determination of ancestral states of alleles, this sample typically fell outside the grouping formed by the NOCA populations, indicating adequate 'rooting' of the phylogenetic tree.

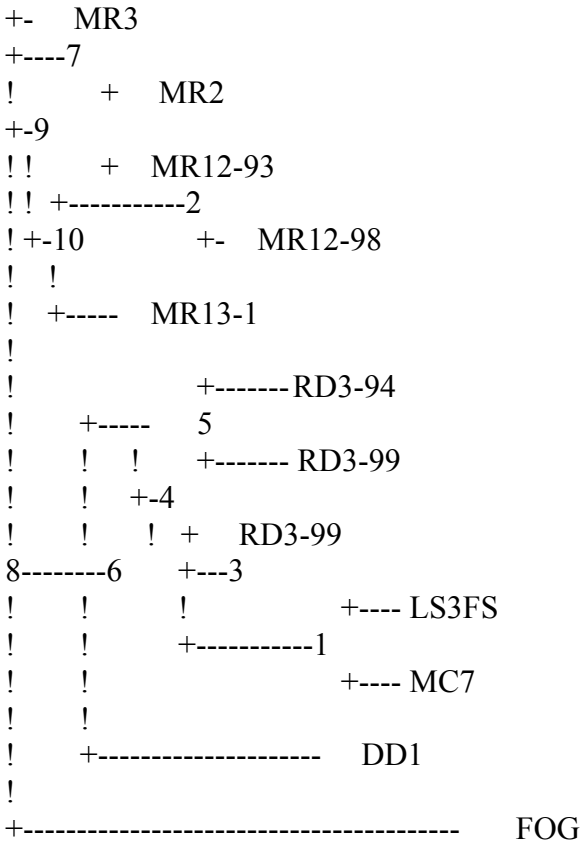

Figure 7. Most frequently-supported dendrogram of genetic relationships among NOCA and Willamette Valley populations of *A. macrodactylum*.

DISCUSSION

The levels of genetic distinction between NOCA and Willamette Valley populations of *A. macrodactylum* in the current study were equivalent to those observed between sub-species or distinct species in other organisms (Shaffer et al. 1991, Larson and Dimmick 1993, Shaffer and McKnight 1996). However, inference about the phylogenetic significance of these results cannot be made in the current study without assessment of additional populations across the geographic range of this species.

Previous genetic studies of high-elevation populations of *A. macrodactylum* documented high within-population genetic variation by examining polymorphic allozyme loci (Funk et al

1999; Tallmon *et al*. 2000), and the current study also documented high levels of heterozygosity and allelic diversity in all populations surveyed. In the absence of selection, genetic diversity in small populations is expected to decline over time in the absence of gene flow due to stochastic effects alone. The persistence of high levels of genetic diversity within each small local population despite low estimated gene flow suggests other mechanisms may be operating to maintain local genetic diversity in high-elevation populations of *A. macrodactylum.*

Table 8. Comparison of past and current studies on population genetics of high-altitude populations of *A. macrodactylum* suggest microsatellites reveal higher degrees of polymorphism.

	Funk et al. 1999	Tallmon et al. 2000	Current Study
Geographic Area	Bitterroot Mountains, ID MT	Bitterroot Mountains, ID MT	North Cascades, WA
Method	Allozymes	Allozymes	Microsatellites
Loci Examined	4	18	8
Loci Polymorphic	4	6	8
Average Alleles per Locus	2.25	1.5	7.67
Range of Observed Average Heterozygosity	0.12 – 0.435	0.04 – 0.14*	0.125 – 0.607
Total Diversity Among Populations	NA	0.299	0.221

* Includes monomorphic loci in calculations of heterozygosity.

Geographic structuring of genetic diversity among populations was evident in this preliminary study. *Ambystoma macrodactylum* populations located on the east slope of the Cascade Range in NOCA were more genetically similar to one another than they were to those located on the west slope, and vice versa. In other words, the genetic differences between the east and west groups or clades were greater than the differences seen among populations within either of these groups, and more significant than the differences seen between lakes with and

without fish. These results are similar to the geographic structuring reported in earlier studies of high-elevation populations of *A. macrodactylum* by Tallmon et al. (2000), who documented hierarchical genetic structuring at the deme, basin, ridge, and larger landscape-levels.

Multivariate space occupied by points generated from samples from east slope populations was more constrained than samples from west slope populations, reflecting the lower levels of genetic diversity, independent of sample size, estimated population size, or the presence or absence of fish. The most parsimonious explanation was the difference in elevation of lakes from which samples were collected in the two drainages. Genetic diversity was negatively correlated with elevation, and all the east slope samples were collected from higher elevation lakes (range 1880-1981m). Previous studies (Funk et al. 1999, Tallmon et al. 2000) did not address the relationship between elevation and genetic diversity.

Populations from the west slope were characterized by higher levels of genetic diversity, even though the average sample size was smaller than those analyzed from populations on the east slope and three of the analyzed samples were from populations sympatric with fish. These samples were all collected from lakes at lower elevations (below 1600m) than east slope lakes.

Another factor that could contribute to genetic differences between east and west slope populations is that the habitats in these areas could have been colonized from two separate refugia or genetically distinct, low elevation source populations located within the Skagit (west of Cascades crest) and Stehekin (east of the Cascades crest) drainages. These separate low-elevation areas could have served as separate glacial refugia and as subsequent sources of colonists for post-glacial dispersal. Unfortunately, we were unable to obtain tissues from individuals from valley floor populations in the Skagit and Stehekin drainages to test this hypothesis.

Fst values indicated that local populations of salamanders within NOCA were genetically unique with low levels of estimated gene flow or migration, as has been reported for other populations of *A. macrodactylum* (Funk et al. 1999, Tallmon et al. 2000). The highest level of migration or gene flow was detected between two populations in very close physical proximity (MR2 and MR3). By assessing gene flow, one can make inferences concerning dispersal rates, routes of dispersal, and degree of isolation of populations (Slatkin 1985, 1987). Rates of migration and movement between local populations generally is positively correlated with the quality of habitat in migration corridors, the lack of significant obstacles, and the size of the habitat patches to which animals are dispersing (Reh and Seitz 1990, Hanski 1991, Sjogren 1991). Rates of migration are generally negatively correlated with distance between populations and degrees of philopatry (Hanski 1991). Close geographic proximity facilitates migration and gene flow among populations, so populations that are in close proximity with few barriers to migration tend to be genetically similar. In NOCA genetic distance was positively related to geographic distance, as has been reported for other populations of *A. macrodactylum* (Funk et al 1999, Tallmon et al. 2000). For NOCA populations of *A. macrodactylum*, we observed the highest estimated levels of genetic similarity and the highest migration rate for the MR2 and MR3 populations. These two lakes are in close proximity to one another, are at approximately the same elevation, and no terrestrial barriers lie between them. However, detected levels of gene flow among most populations of *A. macrodactylum* in NOCA were low and were unrelated to geographic distance. This finding is consistent with the highly philopatric nature of this species, the small size of local populations as sources of migrants, the ruggedness and hostility of the intervening terrain between lakes, and the brief season available for terrestrial dispersal each year.

Previous genetic studies of *A. macrodactylum* documented very low levels of gene flow by examining six polymorphic allozyme loci (Tallmon et al. 2000). However, other studies by the same researchers have suggested that recolonization of lakes can occur within a relatively short time period (within 20 years) after removal of introduced trout from lakes (Funk and Dunlap 1999). Funk and Dunlap (1999) do not resolve these apparently conflicting results. If indeed rapid colonization can occur despite low detected levels of gene flow, this suggests that interpopulation dispersal may not result in gene flow because migrants are unsuccessful at reproduction after dispersal to occupied habitats. Since colonists are apparently able to found new populations in the absence of established conspecific competition, the observed colonizations by Funk and Dunlap (1999) would be consistent with the phenomenon of relaxed selection for early migrants into unoccupied habitat.

Another explanation, however, for these apparently conflicting data would be the recovery of extremely depressed local populations after the removal or extinction of trout. Careful examination of the methods used by Funk and Dunlap (1999) to assess the presence of salamander larvae suggest low densities of salamander larvae in initial assessments may have been undetected. Presence or absence of salamander larvae during the initial (1978) survey of the study lakes was determined solely from shoreline visual surveys designed to confirm the presence of trout, and the shoreline survey was terminated at each lake when the presence of trout was confirmed. Funk and Dunlap (1999) state "…only those lakes without trout were thoroughly surveyed for salamander larvae. No salamander larvae were ever seen in lakes containing trout, but because the entire perimeters of these lakes were not searched, it is not known whether or not salamander larvae were actually absent from all of these lakes." In contrast, their surveys in 1997 and 1998 were designed expressly for the detection of

salamanders, and started with careful shoreline surveys of the entire perimeter of all but two of their study lakes. In addition, these surveys employed snorkel surveys when shoreline surveys failed to detect salamander larvae. They concluded rapid colonization by *A. macrodactylum* had occurred in all lakes where salamander larvae were detected in the 1997-98 surveys but not in the 1978 surveys. Funk and Dunlap (1999) state that the later snorkel surveys were always concordant with shoreline surveys for detecting the presence or absence of salamander larvae, thereby justifying shoreline surveys alone were "…sufficient for determining the presence or absence of salamanders and trout…"

Ambystoma macrodactylum larvae are capable of surviving in lakes with fish, albeit at low densities (Tyler et al. 1998a, this study). Tyler et al. (1998a, b, 2001) found that *A. macrodactylum* larvae tended to remain hidden in substrate materials when trout were present, making them especially difficult to detect from the shoreline. Thus, small populations of *A. macrodactylum* could have been present in the 1978 survey cited by Funk and Dunlap (1999) by were undetected by shoreline viewers. These small populations could have increased and larvae could have been more visible after the removal of trout

The frequency of colonization of new lakes or re-colonization of lakes after local extinction events in NOCA would be predicted to be low for several reasons. First, low levels of gene flow were detected in this preliminary study. Second, most lakes are small, representing small 'targets' of suitable habitat for colonists to find in a hostile landscape matrix. Third, many lakes suitable for *A. macrodactylum* reproduction in NOCA are at high elevation and isolated from other such lakes by significant linear distance, elevation changes, and various features of the landscape and topography that present major barriers to terrestrial dispersal by adult salamanders during a brief snow-free season each year. Fourth, the amount of time available for

terrestrial colonization in high elevation areas is relatively short (a few months).

Another factor reducing the frequency of establishment of self-sustaining populations in new lakes is the obligate mode of sexual reproduction characteristic of this species. New populations are easily established by single individual colonists of species that reproduce clonally or asexually, but this is a rarer event in organisms that reproduce sexually (Hanski 1991). Although fertilization is internal in *A. macrodactylum*, egg deposition usually occurs within a short period of mating, and is usually within the same body of water where mating occurred. Colonization by a single gravid, fertilized female would be limited to the distance a female could travel between mating and oviposition, requiring close proximity of source populations for colonization to occur this way. Successful establishment of a reproductive population would likely require immigration by at least one male and one female. Since most documented populations are small, they probably do not represent major 'source' populations, and would not be expected to send out large numbers of colonists due to density-dependent dispersal. Although colonization might occur rapidly in low elevation lakes in close proximity to other larger populations, the frequency of colonization would be expected to decline with increased lake elevation, decreased lake size, and increased distance from other populations. In light of nearly ten years of work on *A. macrodactylum* in NOCA (Tyler et al 1998a,b, Liss et al 2002), we do not recommend assessing presence or absence of *A. macrodactylum* solely by observation from the shoreline.

We were unable to definitively document major genetic impacts from introduced trout on the native salamander populations in this preliminary study. In regression analyses the presence of fish was not a significant variable in explaining differences among lakes in genetic diversity, and when added to the regression equations contributed very little to explanation of differences

in genetic diversity among populations. Nor were there significant differences in genetic diversity between salamanders in lakes with fish and fishless lakes. Reductions in genetic diversity that were observed in MR 12 after introduction of fish also were observed in a fishless lake, RD3, between time periods. Thus distinguishing genetic impacts of stocked trout from impacts resulting from natural stochastic events (failed reproduction due to a series of severe winters, heavy snowfall, late springs, and resultant short growing seasons) could prove difficult. Resolution of this issue will require additional comparisons of a large number of samples from a large number of lakes over a longer time period.

Storfer and Sih (1998) found that local adaptation of effective predator avoidance behaviors in salamanders was easily swamped by gene flow from individuals dispersing from fishless populations, where selection favored salamanders that were active, diurnal foragers who ate freely and grew and metamorphosed rapidly (Werner and Anholt 1993). Thus, high rates of dispersal among local populations would be expected to prevent the evolution of local populations adapted for coexistence with introduced fishes. This phenomenon may not be a major factor influencing genetic diversity in lakes with non-reproducing fish in NOCA because migration rates among NOCA salamander populations apparently are low. If sufficient genetic diversity exists and persists as the raw material for natural selection in local salamander populations, long-term studies may be useful for testing the prediction that salamander populations sympatric with introduced trout in NOCA will eventually evolve locally-adaptive gene pools for cryptic coloration and behaviors that are compatible with coexistence with diurnal piscine predators.

LITERATURE CITATIONS

Avise, J.C. 1994. Molecular markers, natural history and evolution. Chapman and Hall, New York.

Bahls, P. 1992. The status of fish populations and management of high mountain lakes in the western United States. Northwest Science 66:183-193.

Belkhir, K., P. Borsa, L. Chikhi, N. Raufaste and F. Bonhomme. 2001. Genetix: Genetix, logiciel sous Windows TM pour la génétique des populations, Laboratoire Génome, Populations, Interactions, CNRS UPR 9060, Université de Montpellier II, Montpellier (France).

Blaustein, A.R. and D.B. Wake. 1990. Declining amphibian populations: a global phenomenon? Trends in Ecology and Evolution 5: 203-204.

Blaustein, A.R., D.G. Hokit, R.K. O'Hara, and R.A Holt. 1994. Pathenogenic fungus contributes to amphibian losses in the Pacific Northwest. Biological Conservation 66: 251-254.

Bradford, D.F. 1989. Allopatric distribution of native frogs and introduced fishes in high Sierra Nevada lakes of California: implication of negative effects of fish introductions. Copeia 1989: 775-778.

Bradford, D.F., F. Tabatabai and D.M. Graber. 1993. Isolation of remaining populations of the native frog, *Rana muscosa*, by introduced fishes in Sequoia and Kings Canyon National Parks, California. Conservation Biology 7(4): 882-888.

Bruford, M.W. and R.K. Wayne. 1993. Microsatellites and their applications to population genetic studies. Curr. Opin. Genet. Dev. 3:937-943.

Burke, T., O. Hanotte, M.W. Bruford, and E. Cairns. 1991. Multilocus and single locus minisatellite analysis in population biological studies. Pp 154-168 in T. Burke, G. Dolf, R. Wolff, and A.J. Jeffreys, eds. DNA fingerprinting: approaches and applications. Birkhaeuser, Basel.

Corkran, C.C., and C. Thoms. 1996. Amphibians of Oregon, Washington, and British Columbia. Lone Pine Publishing, Edmonton, Canada.

Fellers, G.M. and C.A. Drost. 1993. Disappearance of the Cascades frog at the southern end of its range, California, USA. Biological Conservation 65: 177-181.

Felsenstein, J. 2001. PHYLIP (phylogeny inference package). Version 3.6. Distributed by the author, Department of Genetics, University of Washington, Seattle.

Funk, W.C. and W.W. Dunlap. 1999. Colonization of high-elevation lakes by long-toed salamanders (*Ambystoma macrodactylum*) after the extinction of introduced trout populations. Can. J. Zool. 77: 1759-1767.

Gresswell, R.E., W.J. Liss, G.A. Lomnicky, E.A. Deimling, R.L. Hoffman, and T. Tyler. 1997. Using mark-recapture methods to estimate fish abundance in small mountain lakes. Northwest Science 71: 39-44.

Hanski, I. 1991. Single species metapopulation dynamics: concepts, models, and observations. Biological Journal of the Linnean Society 42: 17-38.

Harris, A.S. and J.M. Wright. 1995. Nucleotide sequence and genomic organization of cichlid fish minisatellites. Genome 38(1): 177-184.

Harrison, S. 1991. Local extinction in a metapopulation context: an empirical evaluation. Biological Journal of the Linnean Society 42: 73-88.

Harrison, S. 1994. Metapopulations and conservation. *In* P.J. Edwards, R.M. May, and N.R. Webb (eds.), Large scale ecology and conservation biology, p. 111-128. Blackwell Scientific Publications, Oxford, UK.

Hartl, D.H. and A.G. Clark. 1997a. Organization of Genetic Variation, Chapter 3 in: Principles of Population Genetics (third edition). Sinauer Associates, Inc. Sunderland , MA.

Hartl, D.H. and A.G. Clark. 1997b. Population Substructure, Chapter 4 in: Principles of Population Genetics (third edition). Sinauer Associates, Inc. Sunderland , MA.

Howard, J.H. and R.L. Wallace, 1981. Microgeographical variation of electrophoretic loci in populations of *Ambystoma macrodactylum columbianum* (Caudata: Ambystomatidae). Copeia 1981(2): 466-471.

Jones, T.R. 1989. The evolution of macrogeographic and microgeographic variation in the tiger salamander, *Ambystoma tigrinum* (Green). PhD Dissertation. Arizona State University.

Lambou, V.W., W.D. Taylor, S.C. Hern, and L.R. Williams. 1983. Comparisons of trophic state measurements. Water Research 17: 1619-1626.

Larson, A., and W. Dimmick. 1993. Phylogenetic relationships of the salamander families: an analysis of congruence among morphological and molecular characters. Herpetol. Monogr. 7: 77-93.

Lewis, P.O., and D. Zaykin. 1996. Genetic data analysis. Vers. 1.0, D6. IBM Software. Albuquerque, NM.

Liss, W.J., R.L. Hoffman R.E. Gresswell, E.A. Deimling, and G.L. Larson. 1995. Effects of land-use activities on lacustrine systems: literature review. Report to the USDA Forest Service, Eastside Ecosystem Management Project, Walla Walla, Washington.

Liss, W.J., Larson, G.L., E.A. Deimling, R. Gresswell, R. Hoffman, M. Kiss, G. Lomnicky, C.D. McIntire, R. Truitt, and T. Tyler. 1995b. Ecological effects of stocked trout in naturally fishless high mountain lakes: North Cascades National Park Service Complex, WA, USA. NPS/PNROSU/NRTR-95-03. National Park Service, Pacific Northwest Region, Seattle.

Liss, W. J., G. L. Larson, L. Gaio, T. J. Tyler, and R. L. Hoffman. 2002. Distribution and abundance of larval salamanders (*Ambystoma macrodactylum*) large diaptomid copepods in relation to introduced trout and abiotic factors. *In* Liss, W. J., G. L. Larson, and R. L. Hoffman (editors). 2002. Ecological impact of introduced trout on native aquatic communities in mountaiin lakes, Phase III Final Report. USGS Forest and Rangeland Ecosystem Science Center, Corvallis, Oregon. 102 pp

McKnight, M.L. and H.B. Shaffer. 1997. Large, rapidly evolving intergenic spacers in the mitochondrial DNA of the salamander family Ambystomatidae (Amphibia: Caudata). Mol. Biol. Evol. 14(11): 1167-1176.

Nei, M. 1972. Genetic distance between populations. Am Nat. 106:283-292.

Nei, M. 1978 Estimation of average heterozygosity and genetic distance from a small numer of individuals. Genetics 89: 583-590.

Olsen, J. B., J. K. Wenburg, and P. Bentzen. 1996. Semiautomated multilocus genotyping of Pacific salmon (*Oncorhynchus spp*.) using microsatellites. Molecular Marine Biology and Biotechnology 5(4): 259-272.

Paloheimo, J.E. and R.R. Fulthorpe. 1987. Factors influencing plankton community structure and production in freshwater lakes. Canadian Journal of Fisheries and Aquatic Sciences 44: 650-657.

Raymond M. and F. Rousset. 1995. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. J. Heredity 86:248-249.

Reh, W. and A. Seitz. 1990. The influence of land use on the genetic structure of populations of the common frog *Rana temporaria*. Biological Conservation 54: 239-249.

Scribner, K.T., J.W. Arntzen, and T. Burke. 1994. Comparative analysis of intra- and interpopulation genetic diversity in *Bufo bufo*, using allozyme, single-locus microsatellite, minisatellite, and multilocus minisatellite data. Mol. Biol. Evol. 11(5): 737-748.

Shaffer, H.B., and M.L. McKnight. 1996. The polytypic species revisited: genetic differentiation and molecular phylogenetics of the tiger salamander (*Ambystoma tigrinum*) (Amphibia: Caudata) complex. Evolution 50: 417-433.

Shaffer, H.B., J.M. Clark, and F. Kraus. 1991. When molecules and morphology clash: a phylogenetic analysis of the North American ambystomatid salamanders (Caudata: Ambystomatidae). Syst. Zool. 40(3): 284-303

Sjogren, P. 1991. Extinction and isolation gradients in metapopulations: the case of the pool frog (*Rana lessonae).* Biological Journal of the Linnean Society 42: 135-147.

Sjogren-Gulve, P. and C. Ray. 1996. Using logistic regression to model metapopulation dynamics: large scale forestry extirpates the pool frog. *In* D. R. McCullough (ed.), Metapopulations and wildlife conservation, p. 111-137.

Slatkin, M. 1985. Gene flow in natural populations. Annu. Rev. Ecol. Syst. 16:393-430.

Slatkin, M. 1987. Gene flow and the geographic structure of natural populations. Science 236:787-792.

Stephens, J.C., D.A. Gilbert, N. Yuhki, and S.J. O=Brien. 1992. Estimation of heterozygosity for single-probe multilocus DNA fingerprints. Mol. Biol. Evol. 9:729-743.

Storfer, A. and A. Sih. 1998. Gene flow and ineffective antipredator behavior in a stream-breeding salamander. Evolution 52(2): 558-565.

Reynolds, J., B.S. Weir and C.C. Cockerham. 1983. Estimation of the coancestry coefficient: Basis for a short-term genetic distance. Genetics. 105: 767-779.

Tallmon, D.A., W.C. Funk, W.W. Dunlap, and F.W. Allendorf. 2000. Genetic differentiation among long-toed salamander (*Ambystoma macrodactylum*) populations. Copeia 1: 27-35.

Taylor, J. 1983. Orientation and flight behavior of a neotenic salamander (*Ambystoma gracile*) in Oregon. American Midland Naturalist 109(1): 40-49.

Titus, T. 1990. Genetic variation in two subspecies of *Ambystoma gracile* (salamanders (Caudata: Ambystomatidae). J. Herpetology 24(1): 107-111.

Tyler, T.J., W.J. Liss, L. Ganio, G.L. Larson, R.E. Hoffman, E.A. Deimling, and G. Lomnicky. 1998a. Interaction between introduced trout and larval salamanders (*Ambystoma macrodactylum*) in high-elevation lakes. Conservation Biology 12: 94-105.

Tyler, T.J., W.J. Liss, R.L. Hoffman, and L.M. Ganio. 1998b. Experimental analysis of trout effects on survival, growth, and habitat use of two species of western ambystomatid salamanders. Journal of Herpetology 32: 345-349.

Tyler, T. J., W. J. Liss, G. L. Larson, L. Ganio, And R. L. Hoffman. 2002. Abundance and
behavior of larval long toed salamanders (*Ambystoma macrodactylum)* in naturally fishless lakes and lakes with non- reproducing populations of introduced trout in the Northern Cascade Mountains, Washington, USA. *In* Liss, W. J., G. L. Larson, and R. L. Hoffman (editors). 2002. Ecological impact of introduced trout on native aquatic communities in mountaiin lakes, Phase III Final Report. USGS Forest and Rangeland Ecosystem Science Center, Corvallis, Oregon. 102 pp

Weir, B.S. and C.C. Cockerham. 1984. Estimating F-statistics for the analysis of population structure. Evolution 38:1358-1370.

Werner, E.E. and B.R. Anholt. 1993. Ecological consequences of the trade-off between growth and mortality rates mediated by foraging activity. Am. Nat. 142:242-272.

Wright, S. 1978. Evolution and the Genetics of Populations. Volume 5. Variability within and among Natural Populations. University of Chicago Press, Chicago.

APPENDIX

Appendix 1. Forward and reverse primer sequences (5' – 3') for each of the twelve microsatellite loci utilized in the current study.

Locus Name	Forward Primer Sequence	Reverse Primer Sequence
F104	CACAGCCTTCAATGTTCAG	CTGGTGACAGTAGACAGTTCAG
F11	CCGGCACTCCTAAATAGTG	TACGGGAAACTCTTCCTGA
F136	TCTTCCGAACAATCTGACTATG	ATGCGGTTCTCAAAAGAGAG
F142	AATCCTGCTCCACATTTAATC	AGGAAGACAATGGAAAGACAC
H18	GGATTTAGCGTGAGGTG	AAAGACAGACAGACATACACAA
H20	TAGGCTCTTCCACTGGTG	TCTTTGTTGTCTGTCTTGTGA
H29	CCTGGCCTACTACCCATAA	AAGCAATCAGTGAAGAACTTTC
H120	GAGAGGTAGAGAGAGGAGATTG	GCAAGAAAATGCCTAACAA
H123b	TGCCATTCTTCCCTATTTG	AACCCACTGACAACTTTGG
H129	GCCAGGATCATTATTGTAAAC	GTGACACACTGGGAACTACA
H136	CGTGGGTTACTGTGTGCC	CCTATGAGTGAGTGGCGCTATA
F12b	TTCTCTGACCCCCAAACTGT	CCTGTGTGAGCCAAGGTGT

Appendix 2. Summary of the number of the numbers and sizes of alleles detected in the NOCA populations and one Willamette Valley population sampled for each of the twelve polymorphic microsatellite loci for which primer pairs were developed. DNA from a total of 218 individuals from eleven different samples was subjected to PCR amplification with primer pairs for each locus. 'Fails' represent samples that failed to yield any amplification product from a total of three polymerase chain reactions with the primers for the given locus. 'Total' at the bottom of the 'N' column indicates the number of individuals for which successful amplifications were scored for alleles for a given locus. Numbers of copies of each allele detected and frequencies of each allele are for the total number of salamander larvae analyzed among all populations and times.

Summary of results for 12 loci

Average number of alleles = 12.5

Locus	F136			F11			F104			H136		
	Fails	22		Fails	22		Fails	6		Fails	8	
	Allele	N	Freq.	Allele	N	Freq.	Allele	N	Freq.	Allele	N	Freq.
	159	17	0.087	156	1	0.005	196	2	0.009	127	4	0.019
	168	10	0.051	163	4	0.020	202	86	0.406	131	5	0.024
	174	147	0.750	168	1	0.005	208	116	0.547	136	55	0.262
	177	2	0.010	172	79	0.403	211	4	0.019	140	6	0.029
	180	2	0.010	175	4	0.020	214	4	0.019	144	4	0.019
	183	1	0.005	178	2	0.010	Total	212		156	46	0.219
	186	2	0.010	182	2	0.010	# alleles	5		160	6	0.029
	189	1	0.005	186	66	0.337				164	2	0.010
	192	1	0.005	189	18	0.092				168	6	0.029
	195	1	0.005	192	1	0.005				172	23	0.110
	201	1	0.005	195	1	0.005				176	28	0.133
	215	11	0.056	198	13	0.066				180	14	0.067
	Total	196		205	2	0.010				184	11	0.052
	# alleles	12		208	1	0.005				Total	210	
				214	1	0.005				# alleles	13	
				Total	196							
				# alleles	15							

Appendix 2, continued

Locus	H129			H18			H20			H29		
	Fails	10		Fails	188		Fails	8		Fails	10	
	Allele	N	Freq.	Allele	N	Freq.	Allele	N	Freq.	Allele	N	Freq.
	202	1	0.005	238	1	0.033	236	1	0.005	191	4	0.019
	206	4	0.019	243	4	0.133	246	2	0.010	195	171	0.822
	210	3	0.014	251	4	0.133	250	16	0.076	199	11	0.053
	214	2	0.010	255	4	0.133	254	14	0.067	204	12	0.058
	218	1	0.005	259	1	0.033	260	1	0.005	208	2	0.010
	222	20	0.096	263	14	0.467	262	3	0.014	212	2	0.010
	226	35	0.168	275	1	0.033	268	7	0.033	220	2	0.010
	230	69	0.332	279	1	0.033	270	8	0.038	232	2	0.010
	234	35	0.168	Total	30		272	5	0.024	240	2	0.010
	238	14	0.067	# alleles	8		274	54	0.257	Total	208	
	242	7	0.034				278	37	0.176	# alleles	9	
	246	13	0.063				282	27	0.129			
	250	2	0.010				286	29	0.138			
	254	2	0.010				290	2	0.010			
	Total	208					308	2	0.010			
	# alleles	14					318	2	0.010			
							Total	210				
							# alleles	16				

Locus	F142			H120			F12b			H123b		
	Fails	6		Fails	22		Fails	6		Fails	6	
	Allele	N	Freq.	Allele	N	Freq.	Allele	N	Freq.	Allele	N	Freq.
	160	3	0.014	282	1	0.005	170	1	0.005	233	1	0.005
	163	2	0.009	286	1	0.005	173	26	0.123	241	23	0.108
	166	23	0.108	288	103	0.526	176	8	0.038	245	5	0.024
	168	5	0.024	291	10	0.051	179	173	0.816	249	123	0.580
	169	1	0.005	294	14	0.071	182	4	0.019	253	6	0.028
	172	57	0.269	298	1	0.005	Total	212		257	10	0.047
	176	4	0.019	300	2	0.010	# alleles	5		261	27	0.127
	179	6	0.028	306	4	0.020				265	6	0.028
	181	82	0.387	314	1	0.005				269	4	0.019
	184	26	0.123	318	2	0.010				273	4	0.019
	185	1	0.005	322	1	0.005				277	1	0.005
	190	2	0.009	328	1	0.005				281	2	0.009
	Total	212		330	1	0.005				Total	212	
	# alleles	12		353	1	0.005				# alleles	12	
				395	4	0.020						
				399	19	0.097						
				402	1	0.005						
				403	23	0.117						
				407	6	0.031						
				Total	196							
				# alleles	19							

Appendix 3. Alleles documented for all study populations at each of eight microsatellite loci included in the analysis.

ID#	(+/-) fish	E/W slope	F104		H136		H129		H20		H29		F142		F12b		H123b	
DD1-1	+	w	202	202	156	156	230	246	278	278	195	199	166	166	179	179	249	261
DD1-2	+	w	202	208	136	180	246	246	278	278	195	199	172	172	173	179	249	261
DD1-3	+	w	202	202	172	172	246	246	278	278	199	199	176	176	179	179	249	249
DD1-4	+	w	202	202	136	172	218	246	278	282	199	199	172	172	173	179	249	249
DD1-5	+	w	202	202	136	156	226	226	278	278	195	199	172	172	173	179	241	249
FOG-1	-	w	208	211	000	000	206	246	272	272	212	232	163	190	176	179	249	281
FOG-2	-	w	208	211	168	184	226	242	260	290	195	195	160	176	179	179	261	277
FOG-3	-	w	196	208	164	164	210	246	270	318	208	220	179	179	176	176	241	269
FOG-5	-	w	208	208	172	172	234	254	262	262	212	220	172	179	176	179	257	257
FOG-6	-	w	208	211	127	127	210	246	270	318	000	000	160	179	176	182	249	273
FOG-7	-	w	202	211	127	127	210	234	268	268	000	000	179	179	176	179	233	261
LS3-FS-1	+	w	202	202	136	176	226	226	274	282	195	195	184	184	173	179	261	269
LS3-FS-2	+	w	202	202	172	176	222	226	268	272	195	195	166	166	173	179	241	261
LS3-FS-3	+	w	202	202	136	172	226	230	274	286	195	195	184	184	173	179	241	261
LS3-FS-4	+	w	202	202	172	180	226	226	274	282	195	195	166	166	179	179	241	257
MC7-1	+	w	202	202	176	180	222	226	274	282	195	204	166	184	173	179	241	249
MC7-3	+	w	202	202	180	184	226	230	268	274	199	199	166	166	173	179	249	253
MC7-4	+	w	202	202	180	184	226	234	272	274	195	195	160	166	173	179	241	241
MC7-5	+	w	202	202	136	136	226	234	268	282	195	195	166	184	179	179	241	241
MC7-6	+	w	202	202	136	140	226	230	268	274	204	204	166	166	179	179	241	261
MC7-7	+	w	202	202	136	180	226	226	272	282	195	195	166	166	173	173	241	249

Appendix 3, continued.

ID#	(+/-) fish	E/W slope	F104		H136		H129		H20		H29		F142		F12b		H123b	
MR12-1	-	E	208	208	156	156	230	234	250	274	195	195	172	172	179	179	249	249
MR12-2	-	E	208	208	156	156	230	234	250	250	195	195	172	172	179	179	249	249
MR12-3	-	E	208	208	156	156	230	234	250	274	195	195	166	169	179	179	249	249
MR12-4	-	E	208	208	156	176	230	234	274	278	195	195	172	172	179	179	249	249
MR12-5	-	E	208	208	156	156	234	234	274	278	195	195	172	172	179	179	249	249
MR12-6	+	E	208	208	156	156	230	234	250	274	195	195	172	172	179	179	249	249
MR12-7	+	E	208	208	156	176	234	234	274	274	195	195	172	172	179	179	249	249
MR12-8	+	E	208	208	156	156	234	234	250	274	195	195	168	168	179	179	249	249
MR12-9	+	E	208	208	156	156	234	234	274	274	195	195	172	172	179	179	249	249
MR13a-0	-	E	202	208	156	160	230	238	254	274	195	195	172	181	179	179	249	273
MR13a-1	-	E	208	208	136	160	230	238	274	286	195	195	172	172	179	179	249	249
MR13a-2	-	E	208	208	136	136	238	238	286	286	195	195	172	172	179	179	273	273
MR13a-3	-	E	208	208	136	156	230	234	274	274	195	195	172	172	179	179	257	257
MR2-D	-	E	208	208	136	144	230	234	250	278	195	195	172	181	000	000	249	261
MR2-E	-	E	208	208	136	136	226	230	254	274	195	195	181	181	179	179	249	249
MR2-F	-	E	208	208	156	176	230	234	274	278	195	195	172	181	179	179	245	249
MR2-G	-	E	202	208	156	176	222	234	274	286	195	195	184	184	179	179	249	257
MR2-H	-	E	208	208	136	140	230	230	250	274	195	195	176	184	179	179	249	249
MR2-I	-	E	208	208	144	176	222	230	278	286	195	195	172	181	179	179	249	249
MR2-J	-	E	208	208	144	156	230	238	254	278	195	195	172	181	179	179	249	261
MR2-K	-	E	208	208	156	184	230	230	274	286	195	195	181	181	179	179	249	249
MR2-L	-	E	208	208	136	168	222	222	274	278	195	195	181	181	179	179	249	249
MR2-M	-	E	208	208	176	184	230	230	254	278	195	195	172	181	179	179	249	249
MR2-N	-	E	208	208	136	176	230	230	274	274	195	195	181	181	179	179	245	249
MR2-O	-	E	202	208	168	168	226	230	250	274	195	195	172	181	179	179	249	249
MR2-P	-	E	208	208	180	184	230	230	278	282	195	195	172	181	179	182	261	261
MR2-Q	-	E	208	208	156	172	230	234	254	278	195	195	184	184	179	179	249	261

Appendix 2, continued.

ID#	(+/-) fish	E/W slope	F104		H136		H129		H20		H29		F142		F12b		H123b	
MR2-R	-	E	202	202	156	172	222	230	254	274	195	195	172	181	179	179	249	249
MR2-S	-	E	202	208	172	172	230	230	254	278	195	195	181	181	179	179	249	249
MR2-T	-	E	208	208	136	172	230	230	254	278	195	195	172	172	179	179	249	249
MR2-U	-	E	208	208	176	184	230	238	246	274	195	195	172	181	179	179	249	249
MR2-V	-	E	208	208	156	156	222	234	278	282	195	195	181	181	179	179	249	249
MR2-W	-	E	208	208	168	168	226	234	246	250	195	195	172	181	179	179	249	249
MR3-10	-	E	208	208	136	176	226	230	274	286	195	195	181	181	179	179	249	249
MR3-11	-	E	202	202	156	180	226	230	250	286	195	195	172	181	179	179	249	249
MR3-12	-	E	202	208	131	156	230	230	286	286	195	195	181	181	179	179	249	249
MR3-13	-	E	208	208	136	156	222	222	278	278	195	195	181	181	179	179	249	253
MR3-14	-	E	208	208	156	172	226	230	274	286	195	195	172	181	179	179	249	249
MR3-15	-	E	208	208	156	184	230	230	278	286	195	195	181	181	179	179	249	249
MR3-16	-	E	202	208	136	172	230	234	254	278	195	195	172	185	179	179	249	249
MR3-17	-	E	208	208	131	172	222	230	286	286	195	195	181	181	179	179	249	249
MR3-18	-	E	208	208	156	160	222	230	278	278	195	195	181	181	179	179	249	253
MR3-19	-	E	202	208	156	156	202	222	250	274	195	195	172	181	179	179	249	261
MR3-2	-	E	208	208	144	176	226	230	270	270	195	195	172	181	179	179	249	249
MR3-20	-	E	208	208	156	156	226	230	270	274	195	195	181	181	179	179	249	249
MR3-25	-	E	208	208	136	172	230	230	250	278	195	195	172	181	179	179	249	249
MR3-26	-	E	202	208	136	184	230	230	274	286	195	195	181	181	179	179	249	249
MR3-27	-	E	202	208	131	172	230	230	254	278	195	195	172	181	179	179	249	249
MR3-28	-	E	208	208	156	156	222	230	274	286	195	195	172	181	179	179	249	253
MR3-3	-	E	202	202	172	180	230	230	000	000	195	195	172	181	179	179	249	249
MR3-4	-	E	208	208	156	156	222	230	254	286	195	195	181	181	179	179	245	249
MR3-5	-	E	208	208	136	172	222	234	254	270	195	195	181	181	179	179	249	249
MR3-6	-	E	208	208	172	184	226	230	274	274	195	195	172	172	179	179	249	249
MR3-7	-	E	202	208	136	156	222	230	274	286	195	195	181	181	179	182	245	249
MR3-8	-	E	202	202	156	172	226	230	250	250	195	195	181	181	179	179	249	249
MR3-9	-	E	208	208	131	156	222	222	250	274	195	195	181	181	179	179	249	249

Appendix 3, continued.

ID#	(+/-) fish	E/W slope	F104		H136		H129		H20		H29		F142		F12b		H123b	
PR-14	-	W	202	208	136	136	206	206	270	282	195	195	181	181	173	179	241	241
PR-15	-	W	202	208	136	176	214	226	282	282	191	195	181	184	179	179	249	261
PR-16	-	W	202	202	136	136	230	246	286	286	195	204	181	184	173	179	241	241
PR-17	-	W	208	208	136	176	230	234	282	286	195	195	172	181	179	179	257	261
PR-18	-	W	202	202	136	136	230	238	000	000	195	195	181	184	173	179	249	261
PR-19	-	W	202	208	140	180	222	242	278	282	195	195	184	184	179	179	253	265
Pyr-1	-	W	202	202	136	140	226	234	278	282	195	199	184	184	179	179	249	249
Pyr-10	-	W	208	208	136	176	230	234	274	282	195	195	172	181	179	179	257	261
Pyr-11	-	W	202	202	136	176	242	242	278	282	195	195	181	181	173	179	241	261
Pyr-12	-	W	202	208	136	180	226	254	274	274	195	204	184	184	179	179	249	261
Pyr-13	-	W	202	202	136	180	226	234	282	286	195	195	000	000	179	179	241	261
Pyr-2	-	W	202	202	136	136	238	242	278	282	191	195	184	184	179	179	249	261
Pyr-3	-	W	202	202	176	176	242	246	282	282	195	195	166	181	179	179	249	261
Pyr-4	-	W	202	202	136	136	226	234	274	286	195	199	166	166	173	179	241	249
Pyr-5	-	W	202	202	136	172	230	238	282	286	195	204	181	181	173	179	261	261
Pyr-6	-	W	202	202	136	176	234	234	278	308	204	204	181	181	173	173	241	249
Pyr-8	-	W	202	202	136	176	214	230	274	274	195	195	168	181	179	179	249	249
Pyr-9	-	W	202	208	176	176	226	238	274	282	195	195	181	184	179	179	241	245
PyrA-10	-	W	202	202	176	176	230	230	274	282	195	195	181	181	173	179	241	249
PyrA-11	-	W	208	214	136	180	226	226	270	286	195	204	172	172	179	179	249	257
PyrA-12	-	W	202	202	160	176	238	250	282	286	195	204	184	184	173	179	249	265
PyrA-13	-	W	202	214	136	140	238	242	278	282	195	195	181	184	173	173	241	265
PyrA-14	-	W	202	202	136	140	234	238	274	274	191	195	166	166	179	179	249	249
PyrA-15	-	W	202	208	160	176	230	234	286	290	195	204	172	172	173	173	265	265
PyrA-16	-	W	208	214	160	176	238	250	274	282	191	204	181	181	179	179	249	261