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Conservation Genetics of the Imperiled Striped Whipsnake in Washington, USA

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Abstract.—**Conservation of wide-ranging species is aided by population genetic information that provides insights into adaptive potential, population size, interpopulation connectivity, and even extinction risk in portions of a species range. The Striped Whipsnake (*Masticophis taeniatus*) occurs across 11 western U.S. states and into Mexico but has experienced population declines in parts of its range, particularly in the state of Washington. We analyzed nuclear and mitochondrial DNA extracted from 192 shed skins, 63 muscle tissue samples, and one mouth swab to assess local genetic diversity and differentiation within and between the last known whipsnake populations in Washington. We then placed that information in a regional context to better understand levels of differentiation and diversity among whipsnake populations in the northwestern portion of the range of the species. Microsatellite data analyses indicated that there was comparable genetic diversity between the two extant Washington populations, but gene flow may be somewhat limited. We found moderate to high levels of genetic differentiation among states across all markers, including five microsatellites, two nuclear genes, and two mitochondrial genes. Pairwise state-level comparisons and dendrograms suggested that Washington whipsnakes are most closely related to those in Oregon, and distinct from Idaho, Nevada, and Utah, approximately following an isolation by distance model. We conclude that Washington populations of whipsnakes have experienced recent isolating events, but they have yet to lose genetic diversity. The longevity and high vagility of the species may provide opportunity for conservation of whipsnakes in the state as long as shrubland habitat is available.**

Key Words.—connectivity; conservation; fragmentation; genetic diversity; isolation; population structure; reptiles

Introduction

Modern molecular methods have revolutionized identification of life-history patterns for cryptic species and provided insight into potential remedies for their recovery (Frankham et al. 2017). Because cryptic species, including many snakes, are difficult to detect, they are often assumed to be secure on the basis of occasional observations. Low detection rates in snakes also causes some species to be understudied and lack phylogeographic information. Although species with small ranges and low vagility tend to be of conservation concern because of their isolation, phylogeographic information is also needed for snake species with large geographical ranges because even wide-ranging species tend to have population genetic structure at spatial scales relevant to conservation decision making (D'Eon et al. 2002; Inger et al. 2015). Further, wide-ranging species and widely dispersing species may be particularly sensitive to habitat fragmentation if they require large continuous habitats or the ability to reach suitable patches of habitat across a landscape to forage, find mates, and locate refugia. Thus, integrating observed life-history information with multi-scaled population genetic parameters can lead conservation practitioners to a realistic understanding of the issues facing highly vagile snake species and potential remedies for these issues.

The Striped Whipsnake (*Masticophis taeniatus*) is widespread throughout western North America, from Washington, USA, to central Mexico, but documented habitat changes and perhaps road mortality and unregulated persecution in the northern part of its range have resulted in population declines and range contraction (Parker and Brown 1973; Hammerson et al. 2007). Commercial agriculture and urban and suburban sprawl have eliminated large areas of native grassland and shrubland habitat for whipsnakes. The vast road networks that accompany this human infrastructure also contribute to fragmenting remaining habitats into smaller, more isolated areas. Some of these habitat remnants are high quality, but others have been insidiously degraded by invasive plants, particularly annual grasses and forbs that colonize the interspaces

Figure 1. Adult Striped Whipsnake (*Masticophis taeniatus*) captured in Washington State, USA. (Photographed by Lisa Hallock).

between native shrubs and perennial bunch grasses. The resulting loss of unvegetated sandy or rocky soils affects the foraging ability of whipsnakes and the abundance of lizards, their preferred prey (Rieder et al. 2010).

Understanding the ecology of the Striped Whipsnake provides an insight into the causes of their current decline and the negative effects that habitat loss, degradation, and fragmentation have on the species throughout its range. Adult whipsnakes are long (76–183 cm total length), slender, mobile snakes (Fig. 1) that occupy large home ranges (Hirth et al. 1969; Parker and Brown 1972; Parker 1976). In Utah, whipsnakes have been observed moving at least 3.6 km from their hibernacula during spring and summer (Hirth et al. 1969). One male was captured in September at a new den located 16.8 km straight-line distance from the den it emerged from in April. High vagility may allow some whipsnake populations to overcome habitat isolation by maintaining gene flow through dispersal thereby rescuing populations that are at risk of extirpation from high mortality or low recruitment, or by colonizing habitat patches after local extirpation. High vagility in whipsnakes, however, suggests they could also be more sensitive to habitat loss and fragmentation compared to species with small activity areas (Mitrovich et al. 2018). Whipsnakes are active foragers and may need to move frequently to maintain predatory advantage or to locate areas with available prey. Available hibernacula may be a limiting resource for whipsnakes, particularly at the northern part of their range, even for a communally denning species. Striped Whipsnakes will overwinter with other snake species such as racers (*Coluber* spp.), gopher snakes (*Pituophis* spp.), and rattlesnakes (*Crotalus* spp.; Parker and Brown 1973). Finally, whipsnake populations may be sensitive to survival of young because their life-history patterns tend toward K-selection because adults are long-lived (up to 20 y) and reproductive rates are low (annual clutch size of 3–12 eggs; Fitch 1970; Parker and Brown 1972; Brown and Parker 1982).

The negative effects of habitat loss and fragmentation are evident at the northern limit of the range of the species in central Washington where a disjunct population was first reported in the 1940s (Slater 1941). Historical accounts suggest the species was never common in the state and only occurred in the semi-arid, low-elevation (< 1,500 m) areas of the central Columbia Basin. A survey conducted by the Natural Heritage Program of the Washington Department of Natural Resources (WNHP) and U.S. Bureau of Land Management (BLM) from 1998 to 2006 detected the species in only two of the 17 historical locations in Washington, indicating that the species may be at risk of extirpation in the state (Hallock 2006). The two remaining occupied sites are located approximately 7.5 km apart, and 291 km and 471 km from the nearest recorded whipsnake observations in Oregon and Idaho, respectively (i.e., based on all known records, including iNaturalist records 2000–2019; https://www.inaturalist.org/). No other observations of whipsnakes in Washington have been reported to the state or to iNaturalist since 1998, with two exceptions. In 2014, a whipsnake was photographed at Frenchman Coulee in Grant County (Adrian Slade, pers. comm.), which was the first documented observation from that vicinity since a specimen was collected in 1956 (Washington Department of Fish and Wildlife [WDFW] database, University of Washington Burke Museum). The location was surveyed extensively over a 2-d period in 2015 but no other snakes or sheds were observed although the area had recently burned. In 2018, a female whipsnake was captured and photographed at Ginkgo Petrified Forest State Park in Kittitas County (Adrian Slade, unpubl. data), which was the first whipsnake documented at the park, and in the county, since the late 1970s (Gary Lentz, unpubl. data). Similar to the 2014 observation, this snake was also found in an area recently burned by wildfire. This location was searched again later in the year when a shed skin was detected (and used in this study). These two snakes, observed in 2014 and 2018, were within 15 km of the last known populations. Given the rarity of the species in Washington, the Striped Whipsnake is listed as a Species of Greatest Conservation Need in the Wildlife Action Plan of Washington State (WA-SWAP; WDFW 2015) and currently a state candidate for potential listing as an endangered, threatened, or sensitive species by the Washington Fish and Wildlife Commission upon completion of a WDFW status review.

The purpose of this study was to provide population genetic information to aid in the conservation of Striped Whipsnakes in the state of Washington. Specifically, we wanted to measure gene flow between the two remaining populations in Washington and to provide a genetic basis for conservation of the Striped Whipsnake in Washington (U.S. Fish and Wildlife Service

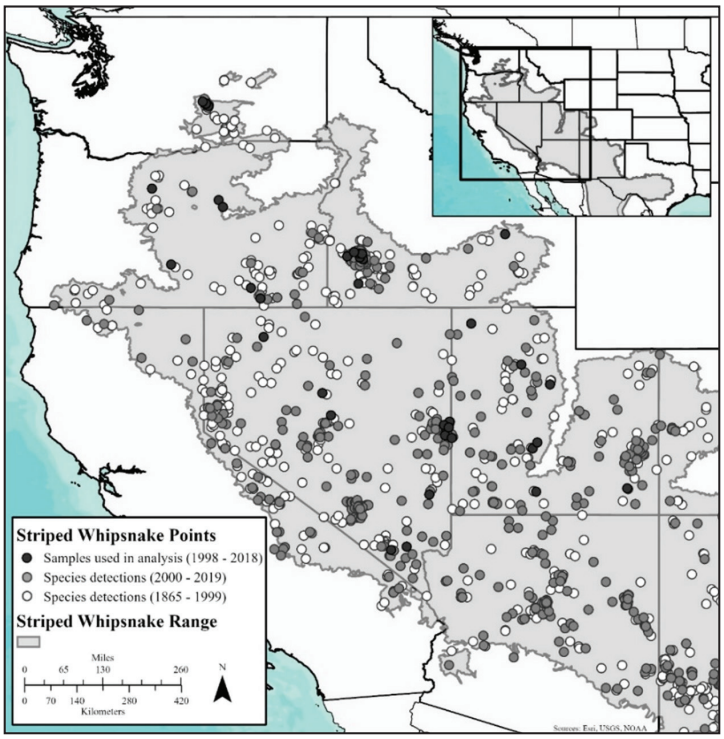

Figure 2. Map of the range and predicted distribution of the Striped Whipsnake (*Masticophis taeniatus*) in the northwestern portion of its range in North America with points illustrating the approximate locations of genetic samples collected. We also display points of captures before and after the year 2000 compiled from museum records and other sources.

[USFWS] - National Marine Fisheries Service [NMFS] 1996). To accomplish these goals, we performed a series of analyses designed to quantify local genetic diversity and differentiation within and between the two extant whipsnake populations in Washington, and place the genetic diversity identified from Washington populations in a regional context to better understand levels of differentiation and diversity among whipsnake populations in the northwestern portion of the range of the species. We hypothesized that Striped Whipsnake genetic diversity is lower at the periphery than at the core of the range of the species and gene flow among populations decreases with increasing geographic distance. We predicted: (1) genetic diversity of the two remaining Washington populations would be lower than populations in the core of the species range (in states further to the south) due to genetic drift in the small, isolated Washington populations (Eckert et al. 2008); (2) the two remaining Washington populations are actually one panmictic population given the short distance between them (7.5 km) and the high vagility of the species (Hirth et al. 1969); and (3) the Washington population would be genetically distinct from its nearest neighboring populations to the south in Oregon, Idaho, Nevada, and Utah because of isolation by distance and reduced connectivity due to human land use (Richmond et al. 2016; O'Connell et al. 2017; Mitrovich et al. 2018).

Materials and Methods

Study area.—Our study area spans the known range of the Striped Whipsnake in the states of California, Idaho, Nevada, Oregon, Utah, and Washington, USA (Fig. 2). More southern and eastern parts of the species range were not included in this study. The species primarily occupies arid Grassland, Desert Shrubland, and rocky canyon habitats, and to a lesser extent Pinyon-Juniper and Pine-Oak Woodlands (Parker 1982; Camper and Dixon 1994; Camper 1996). The species generally occurs below 1,540 m but has been observed in Pinyon-Juniper communities up to 3,077 m in Inyo County, California (Nussbaum et al. 1983; Stumpel 1995; Morrison and Hall 1998). In Washington, habitat for the two extant populations of whipsnakes is dominated by Big Sagebrush (*Artemisia tridentata*) with an understory of bunchgrasses, Cheatgrass (*Bromus tectorum*), and sparse native and exotic forbs. These remaining populations of whipsnakes in Washington, however, occur in remnant sagebrush patches that are at risk of habitat degradation. The occupied Sagebrush-Steppe habitat is surrounded by areas that have been converted to agriculture or inundated by reservoirs when dams were constructed on the Columbia River to provide power and irrigation for agriculture (Simonds 1998). Invasive species, such as Cheatgrass, are abundant and

Table 1. Striped Whipsnake (*Masticophis taeniatus*) shed skins detected at two remaining extant populations in Washington, USA, from 2006–2014. Only 161 of the 163 samples detected at site two were used in genetic analyses because two sheds were too deteriorated to yield DNA. Dashes indicate years when site one was not surveyed.

Sites	2006	2007	2008	2009	2010	2011	2012	2013	2014	Total
Site one	1	3	6	4	—	1	—	1	2	18
Site two	9	21	23	20	24	16	22	15	13	163
Total	10	24	29	24	24	17	22	16	15	181

increase the risk of wildfire as well as decrease habitat quality and landscape connectivity for whipsnakes (Hall et al. 2009). A complete description and map of the study sites in Washington was not provided to protect these sensitive locations.

Sample collection.—In Washington, visual encounter surveys and the collection of shed skins were part of annual surveys of the two remaining extant populations of whipsnakes from 2005 through 2017. Personnel of WNHP led these efforts from 2005–2008 with support from BLM, and personnel of the WDFW led the survey efforts from 2009–2017. Extensive surveys in 2005 yielded five live snakes, but eight sheds were readily detected near apparent hibernacula. This observation led to the current study of using shed skins as an index of snake abundance and a source of DNA for population genetic studies. We collected shed skins for genetic analysis at the southern site (hereafter site one) in 2006–2009, 2011, 2013–2014 and at the northern site (hereafter site two) from 2006–2014 (Table 1). During these surveys, the entire skin was removed from the site to avoid re-sampling the same individual, although the skins of some individuals may have been collected over multiple years because individuals were not identifiable from unique markings. We attempted to control for this issue through individual genotyping (see Data Set Assembly). During the surveys of the two remaining Washington populations, from 2006–2014, we collected 181 shed skins, including 18 samples from site one and 163 from site two (Table 1). We did not use two samples from site two in genetic analyses because of their poor condition. One sample was also collected from an individual as epithelial cells from mouth swabs during a radio-telemetry study in 2006 (Hallock 2006) and an additional shed skin was sent to us from Kittitas County, Washington, in 2018 (Adrian Slade, unpubl. data). We preserved the tissue samples in 95% ethanol, and we stored the collected skins in individual plastic bags at room temperature or at 4° C until the DNA could be extracted for analysis.

We requested all previously collected Striped Whipsnake tissue from the states of Arizona, California, Colorado, Idaho, Nevada, Oregon, Utah, and Washington for evaluation of genetic variation between the remnant Washington populations and surrounding whipsnake populations. We did not include samples from Arizona, California, and Colorado because they had fewer than five samples available, our threshold for inclusion in the study. Tissue was contributed by herpetologists that had collected specimens or samples for previous studies or in the form of roadkill or shed skins. Few samples were available from nearby Oregon sites (Tables 2 and 3) even though we made multiple visits to most historical locations and reached out to all herpetologists working (or having recently conducted field research) in the state.

DNA extraction.—We extracted total genomic DNA from 259 samples: 184 from Washington and 75 samples from neighboring western states. We used an approximately 3 mm^2 piece of shed skin from the tail or a 1 mm^2 piece of tissue for extractions. We extracted the DNA from samples using the DNeasy Blood and Tissue kit (Qiagen, Inc) using the standard kit protocol with the following modifications. We first soaked all samples overnight in sterile ddH_2O to remove surface contamination and any potential inhibitors that might be present. We then performed a preliminary proteinase-K incubation of 20 µl (20 mg/ml) overnight at 56° C. These prepared samples were then macerated with plastic mortars, and incubated again overnight with an additional 20 µl of proteinase-K. After a standard extraction protocol, we eluted samples in 100 ml of Qiagen AE buffer pre-heated to 37° C after incubation at room temperature for 5 min. We extracted all samples in a separate clean laboratory within a containment hood to minimize contamination.

Microsatellite data generation.—We first screened over 50 previously isolated microsatellite loci from five related species for potential use in our study. The majority of these were non-informative monomorphic loci and were not used in further screening. For the final analysis, we used five microsatellite markers (Mle: A114, B107, D11, A109, B105) developed for the Alameda Whipsnake (*Masticophis lateralis*), also known as the Alameda Striped Racer (*Coluber lateralis euryxanthus*; Richmond et al. 2016) for amplifications for 237 (of our 259) whipsnake samples. Amplifications used forward PCR primers labelled with 5' 6-FAM. We performed all PCR reactions in 10 µl volumes consisting of 3 µl diluted DNA extract, 1X PCR buffer, 2 mM $MgCl_2$, 10 mM dNTPs, 0.5 mM each of regular forward and reverse primer, 0.05 mM dye labeled forward primer, and 0.05 U Go Taq Flexi (Promega). The cycling conditions

were 2 min for initial denaturation at 94° C, followed by 35 cycles of 30 s denaturation at 94° C, 30 s annealing at 52°–53° C, and 30 s elongation at 72° C, with a final elongation at 72° C for 10 min. We performed fragment analysis on an ABI 3730 capillary DNA automated sequencer and sized with LIZ 500 standard. All PCRs included blank controls to monitor for contamination. We visualized and scored microsatellite peaks in Geneious v.8.0.2 (Kearse et al. 2012).

We screened microsatellite data for the presence of null alleles and scoring errors using Micro-Checker (van Oosterhout et al. 2004). No evidence for scoring errors existed; however, evidence for potential null alleles existed at loci D11 and A109 at site two. We retained these loci in analyses due to the limited number of loci available for analysis. We also conducted tests for Hardy-Weinberg Equilibrium at each locus for our Washington samples (Supplemental Information Table S1; Waples 2015).

DNA sequence data generation.—For our Washington study sites, we sequenced all 18 individuals from site one and randomly subsampled 20 individuals of the 164 samples from site two. We generated sequences from all samples from surrounding states. We sequenced four genes to produce approximately 2,450 bp of sequence for each sample. Sequencing consisted of two mitochondrial genes (cytochrome B, cytB and cytochrome c oxidase subunit I, COI), and two nuclear genes neurotrophin (NT3) and oocyte maturation factor mos (Cmos; Supplemental Information Table S2). For cytB, we obtained a 950 bp portion of the gene using the primers L14910 and H16064 (Burbrink et al. 2000). The COI gene was amplified using the primers LCO1490 and HCO2198 (Folmer et al. 1994), which have been shown to consistently produce a 700 bp fragment across a broad range of vertebrates. Amplification of the nuclear gene Cmos was accomplished using the primers S77 and S78 (Lawson et al. 2005). These primers were specifically developed to produce an approximately 550 bp fragment in the suborder Serpentes. A 450 bp fragment of the NT3 gene was amplified using the primers NT3FB and NT3RB (Supplemental Information Table S2), which were designed and developed for this study from sequence generated from amplifications from the primers NT3F and NT3R, respectively (Noonan et al. 2006).

We performed Polymerase Chain Reactions (PCR) in 20 µl volumes with AmpliTaq Gold (ThermoFisher Scientific) using standard conditions. Three microliters of diluted DNA extract were used for amplifications. The cycling conditions included a 5 min initial denaturation at 95° C, followed by 35 cycles of 30 s denaturation at 95° C, 30 s annealing at 51°–55° C depending on the primers used, and 1 min elongation at 72° C, with a final elongation at 72° C for 10 min. Negative controls were included with all PCR reactions to monitor for contamination. PCR products were extended and bidirectional sequenced using ABI Big Dye sequencing chemistry on an ABI 3700 automated DNA sequencer. We assembled and aligned sequences for each individual sample in Geneious v.8.0.2 (Kearse et al. 2012). We visually inspected sequences before trimming to a final length.

Data set assembly.—Many samples, particularly from Washington, were based on shed skins that were collected over a period of years as opposed to samples from marked live animals. Therefore, we could not rule out the possibility that individuals were sampled in our data set more than one time. Indeed, of the 232 samples that were genotyped using microsatellite markers, 57 samples had multilocus genotypes that were identical to another sample from the data set. These 57 samples could be assigned to 24 unique profiles (Supplemental Information Table S3) and thus could reflect the resampling of the same individuals over time. Twenty-two of these individuals came from Washington and two came from a location in southeastern Idaho. To evaluate the likelihood that these 24 unique profiles represented 24 individual animals that deposited sheds over time (i.e., over several months or years), we calculated the probability of identity (PID) for each locus and across all loci using Gimlet version 1.3.3 (Valière 2002). The overall PID values were low (0.0013 and 0.0015 for data sets with duplicates included and duplicates removed, respectively) suggesting a reasonable likelihood that these multilocus genotypes belonged to the same individual and were recaptures.

To further overcome the challenges of working with shed skins, we approached analyses of the microsatellite data in two ways to bracket the different potential outcomes. First, we performed analyses that assumed that each shed skin reflected a unique individual that was retained in all of the analyses described below. Second, we performed analyses where only a single representative from each of the 24 multilocus genotypes was retained using the following criteria.

When all genetically identical samples originated from the same population (18 cases), we retained a single representative of that genotype with one exception. This specific exception occurred at site two in Washington where a shed skin from a juvenile that was collected in 2008 had the same multilocus genotype as an adult shed sampled the previous year, thereby precluding the existence of a single individual (but indicating a possible parent-offspring pair). In this specific case, two instances of the multilocus genotype were preserved. Therefore, we considered two duplicate genotypes in Idaho and 16 duplicate genotypes in Washington as

Table 2. Genetic diversity in Striped Whipsnakes (*Masticophis taeniatus*) based on analyses of microsatellite data at two locations in Washington and across states of the USA. Duplicate multilocus genotypes were either included or excluded based on criteria elaborated upon in Methods. Abbreviations are n = sample size, Ho = observed heterozygosity, He = expected heterozygosity, Fis = inbreeding coefficient, A = observed average number of alleles per locus, and Ar = a rarefied estimate of the average number of alleles per locus that accounted for differences in sample size among groups.

Dataset	Site or State	n	Ho	He	Fis	A	Ar
Duplicates included	Site one	18	0.5353	0.4580	-0.1800	3.2	3.2
	Site two	161	0.4894	0.5153	0.0265	7	4.06
Duplicates removed	Site one	14	0.5747	0.4955	-0.1751	3.2	3.2
	Site two	137	0.4872	0.5244	0.0433	7	3.92
Duplicates included	Washington	180	0.4927	0.5136	0.0184	7.4	3
	Oregon	6	0.5417	0.6894	0.2308	3.75	3.6
	Idaho	25	0.3930	0.4739	0.1724	4.2	3.13
	Nevada	13	0.4167	0.6641	0.3751	7.2	4.98
	Utah	8	0.3571	0.6936	0.4787	5.4	4.89
Duplicates removed	Washington	151	0.4957	0.5248	0.0290	7.4	2.47
	Oregon	6	0.5417	0.6894	0.2308	3.75	2.65
	Idaho	22	0.3736	0.4744	0.2155	4.2	2.49
	Nevada	13	0.4167	0.6641	0.3751	7.2	3.39
	Utah	8	0.3571	0.6936	0.4787	5.4	3.47

multiple captures of non-dispersing individual snakes because they were found at the same location over multiple years. Of these 16 cases in Washington, one duplicate genotype was always observed at site one and 15 duplicate genotypes were always observed at site two. In the cases of duplicate genotypes detected at different locations over time (see below), we retained population labels associated with the oldest sample collected (i.e., the presumed site of origin; Table 2).

Given our subsampling associated with site two in Washington for the state-level analysis, far fewer individuals were analyzed for the DNA sequence data sets (Table 3) relative to the microsatellite data (Table 2). Of these individuals, however, relatively few had identical multilocus genotypes (Cmos: eight individuals from three groups; NT3: seven individuals from three groups; cytB: four individuals from two groups; COI: four individuals from two groups). Given the small number of individuals involved relative to the overall size of the data set, we elected to retain all samples in analyses, recognizing the minimal impact that potential duplication may have on analysis outcomes given the broader objectives of the study.

Data analyses.—We analyzed data at two levels: comparisons of site one and site two from Washington, and comparisons of the aggregated sets of samples from each state. In the latter case, we aggregated all 38 Washington samples into a single population, including an additional shed skin collected at Ginkgo Petrified State Forest in 2018 (away from sites one and two). We tested for population genetic differentiation (F_{ST}) among microsatellites between our two Washington populations using the AMOVA procedure (Excoffier et al. 1992) as implemented in Arlequin (Excoffier and Lischer 2010). The significance of genetic differentiation measures was derived based on a randomization procedure comprised of 10,000 randomization replicates. In addition to a global measure of genetic differentiation for each data set, we also calculated all pairwise estimates of F_{ST} among populations to better identify the magnitude and variation of differentiation at this scale. We also used Arlequin to quantify microsatellite genetic diversity based on observed heterozygosity (Ho) and expected heterozygosity (He; Supplemental Information Table S1) and to perform tests for Hardy-Weinberg equilibrium at each locus. We obtained a rarefied estimate of the average number of alleles per locus (Ar), which accounted for differences in sample sizes among populations (Kalinowski 2004), for each population and each data set using the program HP-Rare (Kalinowski 2005). We used the program NeEstimator version 2.1 (Do et al. 2014) to estimate effective population sizes for Washington sites one and two based on the linkage disequilibrium (LDNe) approach outlined in Waples and Do (2008). Because low-frequency alleles can influence point estimates, we obtained separate estimates by excluding alleles with frequencies < 0.05, 0.02, and 0.01, and under a separate situation where all alleles were included regardless of their frequency.

We performed a similar suite of analyses for each of the four genes included in the study for all states with a

Table 3. Genetic diversity estimates for Striped Whipsnakes (*Masticophis taeniatus*) based on analyses of two nuclear genes (Cmos and NT3) and two mitochondrial genes (COI and cytB) for the two remaining sites in Washington and each state. The state-level diversity for Washington includes an additional shed skin collected at Ginkgo Petrified State Forest in 2018 (away from sites one and two). Abbreviations are n = sample size, H = haplotype diversity, π = nucleotide diversity, A = observed number of haplotypes, and Ar = number of haplotypes correcting for differences in sample size among groups.

Gene	Site or State	n	H	π	A	Ar
Cmos	Site one	18	0.111	0.0002	2	2.00
	Site two	20	< 0.001	< 0.0001	1	1.00
	Washington	39	0.051	0.0001	2	1.15
	Oregon	6	< 0.001	< 0.0001	1	1.00
	Idaho	20	0.479	0.0009	2	1.96
	Nevada	11	0.691	0.0023	3	2.80
	Utah	7	0.524	0.0011	3	2.71
NT3	Site one	18	0.765	0.0065	7	7.00
	Site two	18	0.699	0.0047	8	8.00
	Washington	37	0.787	0.0064	15	3.97
	Oregon	6	0.600	0.0030	3	3.00
	Idaho	25	0.870	0.0072	11	4.50
	Nevada	11	0.964	0.0122	9	5.45
	Utah	7	0.952	0.0098	6	5.29
COI	Site one	13	< 0.001	< 0.0001	1	1.00
	Site two	18	0.569	0.0014	7	5.33
	Washington	32	0.345	0.0008	7	2.13
	Oregon	6	0.733	0.0016	3	3.00
	Idaho	17	0.331	0.0009	4	2.06
	Nevada	12	0.742	0.0072	5	3.54
	Utah	7	0.810	0.0019	4	3.71
cytB	Site one	13	0.513	0.0009	3	3.00
	Site two	17	0.420	0.0004	4	3.49
	Washington	31	0.450	0.0007	6	2.54
	Oregon	6	0.800	0.0013	4	4.00
	Idaho	7	0.952	0.0033	6	5.29
	Nevada	11	0.709	0.0037	5	3.45
	Utah	7	0.667	0.0015	3	2.86

sample size of more than five individuals per state. We calculated global and pairwise genetic differentiation (F_{ST}) measures using Arlequin as described above, using the proportion of mismatched nucleotides between haplotypes as the DNA sequence divergence measure. Genetic diversity measures calculated for the DNA sequence data sets included haplotype diversity (H) and nucleotide diversity (π), calculated in Arlequin, along with the rarefied measure of the number of haplotypes (Ar) as calculated by HP-Rare as described above. We tested for isolation by distance patterns based on inter-individual genetic and geographic information using Mantel tests (Mantel 1967) implemented in the program Alleles in Space (Miller 2005) using 1,000 randomization replicates. We performed analyses separately for the microsatellite data and for each of the four DNA sequence data sets.

We generated haplotype networks using the program POPART (Leigh and Bryant 2015) based on the Median-Joining procedure as described by Bandelt et al. (1999) to obtain a graphical description of the relationships among haplotypes for each gene and the degree of haplotype sharing among populations. We also used pairwise F_{ST} matrices calculated by Arlequin for each data set to construct Unweighted Pair Group Method with Arithmetic mean (UPGMA) dendrograms that provided a graphical depiction of the degree of differentiation of the aggregated sets of samples from each state. UPGMA is a hierarchical clustering method. We constructed and visualized UPGMA dendrograms using MEGA-X (Kumar et al. 2018).

Six of the 24 cases where we identified genetically identical multilocus genotypes from microsatellite data came from sheds collected at different Washington sites, indicating potential dispersal. We quantified dispersal distances under the assumption that the identical multilocus genotypes reflected recaptures of individuals over time. The PID values that we calculated (described earlier) support our assumption that these identical multilocus genotypes derived from sheds represent recaptured individuals.

Results

Analyses of the microsatellite data indicated that there was comparable genetic diversity between the two extant Washington populations (Table 2). The number of alleles per locus (A) was lower for site one compared with site two, but this difference mostly disappeared when differences in sample size between groups (Ar) was accounted for (Table 2). Inclusion or exclusion of samples with identical multilocus genotypes (because of sample uncertainty from using shed skins as opposed to marked individuals) had minimal impact on the analysis outcomes or their interpretations. Several loci demonstrated significant deviations from Hardy-Weinberg equilibrium (Supplemental Information Table S1), although results depended in part on whether or not duplicates were included or excluded from analyses. Deviations were more common at site two, possibly due to the greater power of tests conducted in that population by virtue of the larger sample size.

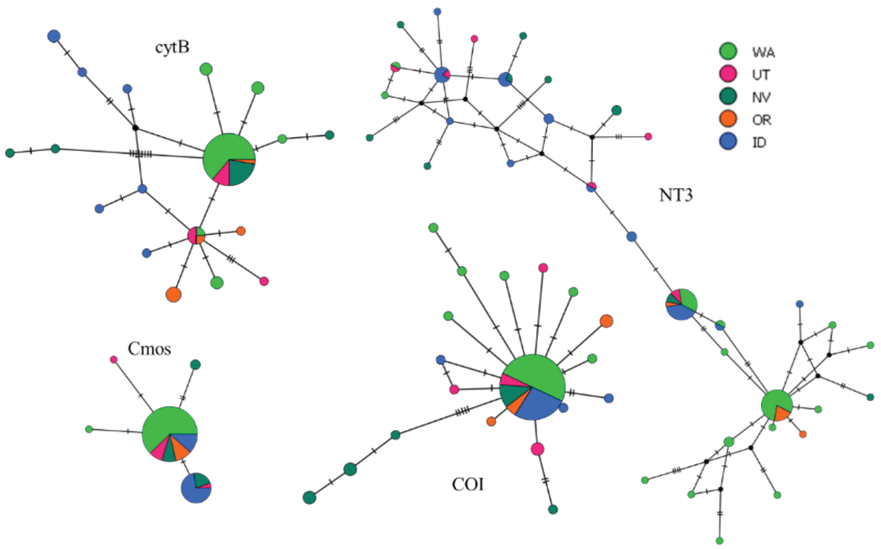

Figure 3. Haplotype networks illustrating relationships among haplotypes detected in four different genes examined for Striped Whipsnake (*Masticophis taeniatus*). The size of each network node reflects the number of samples that possessed each haplotype. Colors depicted on the pie charts for each node indicate populations where each haplotype was observed. State abbreviations are Idaho = ID, Nevada = NV, Oregon = OR, Utah = UT, and Washington = WA.

Results from the DNA sequence data sets were highly variable among the four genes that were examined (Table 3). Haplotype networks based on the different DNA sequence data sets showed little concordance in topology and limited geographic structure (Fig. 3). Collectively, the results point to the presence of moderate levels of diversity among sample groupings; however, the variability among data sets precluded qualitative statements that labeled populations as having low or high genetic diversity (Table 3). Despite the variation among data sets, a common pattern that emerged highlighted the high degree of haplotype sharing among groups (Fig. 3). In all four data sets, one or two high-frequency haplotypes were detected in all populations examined.

Despite the presence of shared haplotypes among groups, we found significant genetic differentiation among the two remaining populations in Washington using analyses of the microsatellite data. This evidence of restricted gene flow was also evident at one of the two nuclear genes examined (NT3). No differentiation was noted at this level for the mitochondrial genes COI or cytB.

We found moderate to high levels of genetic differentiation among states across all markers, including microsatellites, nuclear genes NT3 and Cmos, and mitochondrial genes COI and cytB (Table 4). Despite identifying similar average differentiation patterns at this spatial scale, pairwise levels of differentiation among samples from different states varied depending on which molecular marker was examined (Fig. 4; Supplemental Information Table S4). For microsatellites, we found strong genetic differentiation of Washington whipsnakes from all other states occupied by whipsnakes, including Oregon, its nearest neighbor where the nearest whipsnake observations are about 291 km away. We found no evidence for significant genetic differentiation of Washington whipsnakes from Oregon whipsnakes at either of the nuclear genes (Cmos and NT3), however, and only one of the mitochondrial genes (cytB, but not COI; Supplemental Information Table S4). Differences, particularly in the mitochondrial genome, resulted in considerable variation among our dendrograms (Fig. 4). We observed, however, some consistency between our microsatellite and nuclear (Cmos and NT3) dendrograms. Tests for isolation by distance

Table 4. Measures of genetic differentiation (F_{ST}) in Striped Whipsnakes (*Masticophis taeniatus*) calculated in five DNA sequence data sets and for different partitions of the data as described in Methods. Abbreviations are WA = Washington and DBS = data by states.

	Microsatellites		Cmos		COI		cytB		NT3	
Populations examined	F_{ST}	*P*-value	F_{ST}	*P*-value	F_{ST}	*P*-value	F_{ST}	*P*-value	F_{ST}	*P*-value
WA - duplicates included	0.047	< 0.001	0.006	0.4741	-0.004	0.5141	0.048	0.1456	0.227	< 0.001
WA - duplicates removed	0.030	0.0325	—	—	—	—	—	—	—	—
DBS - duplicates included	0.253	< 0.001	0.394	< 0.001	0.257	< 0.001	0.299	< 0.001	0.335	< 0.001
DBS - duplicates removed	0.243	< 0.001	—	—	—	—	—	—	—	—

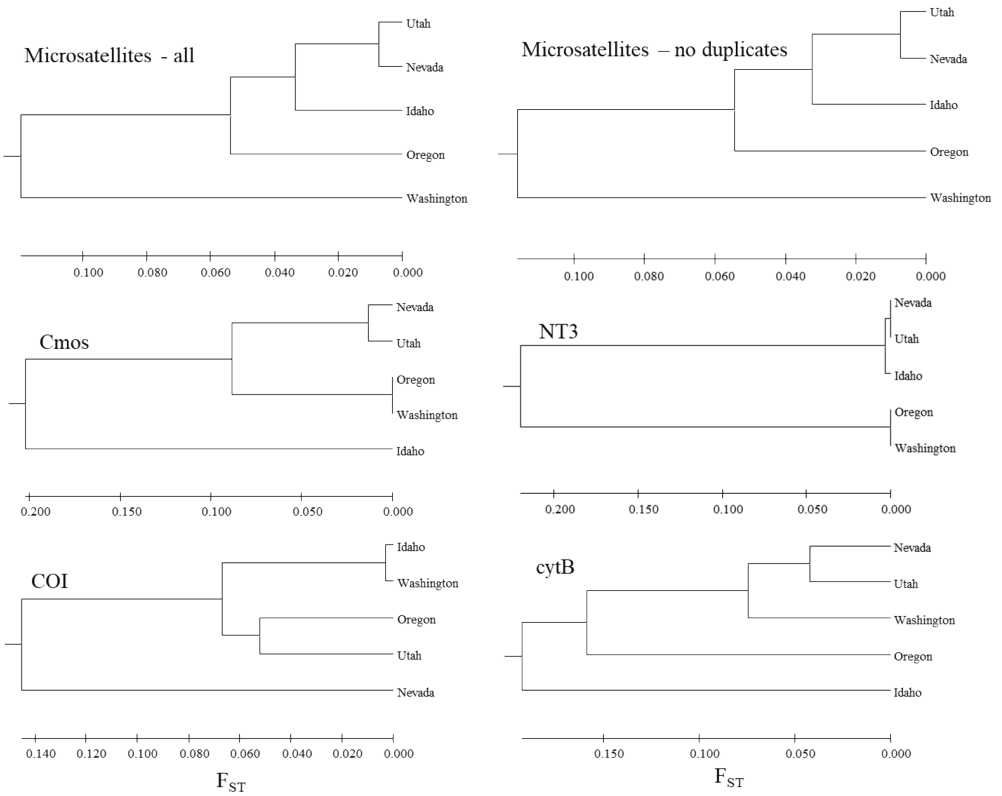

Figure 4. Unweighted Pair Group Method with Arithmetic mean (UPGMA) dendrograms generated based on matrices of pairwise F_{ST} estimates and a measure of genetic differentiation among Striped Whipsnake (*Masticophis taeniatus*) samples collected in five western U.S. states. Dendrograms are presented for each of the four DNA sequence data sets (nDNA: Cmos and NT3; and mtDNA: COI and cytB) produced in this study along with two dendrograms produced for the nuclear microsatellite data using the data set variants described in the methods.

patterns were largely consistent across data sets. With the exception of the cytB DNA sequence data sets, significant correlations between inter-individual genetic distances and geographic distances were detected (Supplemental Information Table S5).

Given the conservation concern for whipsnakes in Washington, we examined two other aspects of their population biology retrospectively: effective population size and dispersal. Using the five microsatellite loci, we found that Ne ranged from 12–55 snakes for site one and 39–134 snakes for site two, depending on whether we included duplicate multilocus genotypes and whether we discarded rare haplotypes (Supplemental Information Table S6). Confidence intervals for these estimates were broad, however, and mostly unbounded (infinite) on the upper end.

We obtained six cases of assumed indirect measures of dispersal from unique multilocus genotypes of shed skin in Washington where two snakes moved south from site two to site one (7.5 km), three snakes moved from site two to locations about 1.6–2.7 km to the south, and one snake moved from site two to a location 7.8 km to the north. This latter snake was last detected at site two on 29 October 2014 before being discovered nearly 8 km away on 11 September 2018. Given that we have no way to determine when a shed skin was deposited relative to when we collected it, however, we are only able to document the time between detections (Supplemental Information Figure S1). In other words, it is just as likely the snake moved that distance quickly in the same year as more slowly over nearly 4 y. For comparison, the snakes that moved south from site two to site one, a similar distance as the snake that dispersed north from site two, were detected 1 and 3 y apart. In most cases, however, detections were separated by 0–1 y (Supplemental Information Fig. S1), probably because we intentionally and effectively removed skins from the sites as we discovered them. A few detections spanned several years, with a maximum of 7 y.

Discussion

This study sheds light on the degree of isolation of the Striped Whipsnake in Washington at the northern limit of its range, an area with documented population extirpations and loss of habitat. Our analysis of within and among population diversity and genetic differentiation revealed considerable variability among

molecular markers, yet some consistent patterns emerged in relation to our proposed hypotheses. To our surprise, however, none of our hypotheses were fully supported by the data.

Contrary to expectations, we found no evidence that isolation of the remaining extant whipsnake populations in Washington has resulted in loss of genetic diversity compared to populations in the core of the species range. Even though these populations are small, we found little evidence for genetic drift at the loci examined. Further, heterozygosity in the Washington populations were generally higher on average than in the southern or core part of the species range (0.357–0.542), a pattern contrary to the central-marginal hypothesis of genetic variation across ranges of species (Eckert et al. 2008). We suspect, however, that genetic diversity in these Washington remnants may begin to decline over time due to our observed restricted gene flow and the few sheds or snakes observed each year despite concerted conservation efforts (Allendorf 1986; Amos and Harwood 1998; O'Connell et al. 2017). Our estimates of effective population size suggest that site one is particularly small, although our estimates had considerable variability. The long generation time in whipsnakes (Pianka and Parker 1975) may delay when genetic effects from fragmentation are detectable because the parents (and grandparents) of the adults we sampled may have experienced habitat conditions and connectivity that were different than today. If reduced genetic diversity does occur, developmental abnormalities and other negative effects that result from inbreeding may begin to be detectable (Gautschi et al. 2002) and could have population-level consequences (Madsen et al. 1996). Given the isolation of these small, remnant snake populations in Washington, we may be at the beginning of an extinction vortex that involves both genetic and demographic effects. A survey of these sites in 2017 revealed six sheds at site one, but only two sheds at site two. If these last remaining populations in the state of Washington continue to decline, complete extirpation may ultimately arrive suddenly because of a harsh climatic event, wildfire, disease, or some interaction of these factors.

We already are beginning to find evidence of genetic differentiation between the two Washington populations. The microsatellite loci examined provided the strongest indication of genetic differentiation. Only one (nDNA: NT3) of the four genes examined was in agreement with this pattern, however, suggesting that gene flow may have been restricted fairly recently given the higher rate of mutation in microsatellites compared to the nuclear and mitochondrial genes examined. We suspect that gene flow may be further limited by recent developments to the area between the sites, such as paving and enlarging a primary road and a recent wildfire that burned much of the intervening shrubland habitat.

Finally, our third prediction that the disjunct Washington population is distinct genetically from populations in the rest of the range was also not fully supported by our findings. Microsatellite UPGMA dendrograms placed Washington as distinct from the other states and potentially suggested an isolation by distance model (and aligned with our hypothesis). The DNA sequence data, however, provided little support for this distinction and generally grouped Washington with Oregon, although distinct from most other states, particularly in the mitochondrial genome. The nuclear genes created dendrograms that were more aligned with our expectations compared to the dendrograms of mitochondrial genes, which aligned Washington with either Idaho (COI) or Nevada and Utah (cytB). The contrasting patterns of mitochondrial and nuclear genetic structure is confusing although not unprecedented and may occur when somewhat isolated populations interact across contact zones (Brito 2007; Richmond et al. 2016). Further, the relative consistency between the microsatellites and the nuclear genes suggest that there may be a genetic consequence of sex-biased dispersal (Hoffman et al. 2006). Mitochondria are only inherited from mothers and thus the lack of evidence for isolation of Washington in the mitochondrial genome may suggest that females are dispersing further than males. Dispersal in whipsnakes is not well understood, especially as it relates to differences between sexes or among age classes (Hirth et al. 1969). Regardless of the mechanism, our data suggest a high degree of haplotype sharing among geographically dispersed samples and thus we found weak evidence for the presence of evolutionarily significant units in Washington relative to surrounding states. We wonder, however, if this is just a matter of time given the rapid decline of whipsnakes in Washington and few observations of whipsnakes in Oregon, especially since 2000 (Supplemental Information Fig. S2).

Our molecular analysis suggests that the two remaining populations in Washington may be undergoing genetic differentiation from each other and from other populations to their south, but only recently. The small size of these two populations is a concern because small populations tend to be more susceptible to loss of genetic diversity or even extirpation. The fact that these two populations retain relatively high heterozygosity, however, suggests that the isolation may be fairly recent and there could be time to promote the conservation of the species in Washington through actions aimed at increasing available habitat and reducing mortality. Protection of hibernacula from persecution, destruction during road improvement and quarrying, and post-fire aeolian sedimentation may be particularly important (Parker and Brown 1973; Brown and Parker 1982).

Our indirect evidence for natural dispersal of at least 8 km from the larger of the last two remaining populations in Washington also provides hope that colonization or recolonization of surrounding habitats is possible. Habitat protection or restoration could aid this process, especially if it provides high quality habitat for whipsnakes and their prey (e.g., lizards), maintains connectivity between remaining populations, and increases dispersal potential such that the species distribution may once again expand in Washington. This habitat protection or restoration, however, has recent urgency. The area between the two populations burned on 4 June 2019. The loss of shrubs in this fire is concerning given how reliant whipsnakes are on shrubs for refugia, foraging, and thermoregulation (Hirth et al. 1969; Brown and Parker 1982). There is also concern that agricultural irrigation, primarily from center pivot, is contributing to observed changes in the surrounding shrubland vegetation communities at the sites. A recently paved road has increased risk of vehicle-related mortality of snakes in the area and a new transmission line has been proposed for one of the bluffs occupied by the species. The Wildlife Conservation Strategy (WCS) of Washington calls for increased efforts to restore habitat on public land, protect sites with easements or agreements, identify specific habitat needs and limiting factors, and develop mitigation strategies. The area currently occupied by Striped Whipsnakes was approved by the Natural Heritage Advisory Council of the state in 2007 as a proposed Natural Area Preserve but none of the parcels within the boundary have been secured.

This research contributes to a broader understanding of the plight of snakes and other reptiles globally (Gibbons et al. 2000; Böhm et al. 2013). An assessment of particularly well-studied snake populations around the world found that the majority are declining since 2000 and some are at risk of extinction (Reading et al. 2010). Researchers suspect climate change may be contributing to these patterns (Araujo et al. 2006; Reading et al. 2010), but there is a general paucity of data on reptile species and populations globally (Tingley et al. 2016). Our investigation into the population genetic diversity and differentiation of whipsnake populations in the northwestern part of the species range provides the type of information needed for many other reptile species. Only through periodic surveys, well-designed monitoring, and research will there be sufficient information for early warning of declines, knowledge of what conservation actions are needed, and time for conservation efforts to succeed. This is challenging for secretive, cryptic species, such as the Striped Whipsnake, but we have demonstrated how indirect measures of detection (i.e., shed skins) combined with molecular methods provided a source of information for conservation of this imperiled species despite its rarity and lack of direct observations or captures.

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