HOST SPECIFICITY, HOST DISPERSAL AND RIVER STRUCTURE: FACTORS AFFECTING PARASITE POPULATION CONNECTIVITY AND THE CONSERVATION GENETICS OF THE CONCHO WATER SNAKE (*Nerodia hateri paucimaculata*)

A Dissertation

By

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ABSTRACT

Estimating gene flow is key to understanding local adaptation, population connectivity and the evolutionary trajectory of a species. For trematode parasites with complex life-cycles, definitive host specificity and dispersal combined with the physical features of landscapes can influence parasite gene flow. Likewise, understanding gene flow for species of conservation concern can help identify genetically isolated subpopulations. In this dissertation, I examine the influence of host specificity, host dispersal and the physical characteristics of the Concho and Colorado Rivers on the population structure of *Renifer ancistrodontis* and *Renifer aniarum* and the conservation genetics of one of their definitive hosts, *Nerodia hateri paucimaculata*. I determined that the host specific parasite, *R. ancistrodontis* exhibited similar levels of genetic diversity and population structure at the local level as the generalist parasite *R. aniarum*. The findings of this study do not support the predicted consequences of host specificity on parasite population structure. I then address the relative influences of host dispersal, unidirectional stream drift and dendritic ecological network on population connectivity of *R. aniarum* in the Colorado and Concho Rivers. I determined that host dispersal largely negates the influence of unidirectional stream drift. *Renifer aniarum* also did not exhibit population structure concordant with the three reaches of the river but neither did it exhibit panmixia among locations. Rather, *R. aniarum* population structure was influenced by a combination of host dispersal and local transmission dynamics with a pattern of isolation by distance associated only with populations located on the distal

branches of the upper reaches of the main-stem river. Finally, I determined that the Concho water snake showed significant population subdivision between the upper and lower Colorado River and O.H Ivie reservoir. Additional local population structure was detected between collection sites within the upper and lower Colorado River. Sibling groups were identified among neonate snakes collected during fall sampling seasons but removal of the siblings did not affect the patterns of population subdivision. Habitat modification and low water flows during periods of drought are likely to contribute to existing patterns of low population connectivity between the upper and lower Colorado River and O.H. Ivie Reservoir.

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CHAPTER I

INTRODUCTION

The amount of gene flow among sub-populations is a primary parameter that determines the evolutionary trajectory and speciation potential of a population. High levels of gene flow among demes will often cause the sub-populations to evolve as a single unit, while low levels of gene flow may allow sub-populations to evolve independently. Parasites represent important systems for investigating population divergence at both the evolutionary and ecological scales as they are one of the most abundant and diverse groups of extant organisms (Poulin and Morand 2004). Additionally, parasites are increasingly recognized as integral components of ecosystems (Wood and Johnson 2015). Parasites have been documented to facilitate cross-ecosystem trophic subsidies, strengthen trophic interactions, alter predation rates and alter ecological community composition (Wood and Johnson 2015). Despite their abundance, diversity and ecological importance, little is known about the factors that can influence population divergence in parasites. This lack of knowledge is due in part to the fact that studies on parasite population biology are hampered by limited morphological traits to correctly identify a species, and the inability to directly observe dispersal or other behaviors of individuals.

Testing the hypothesis of panmixia can provide valuable insight into the ecology and evolution of organisms whose life history or morphology can render direct methods used to test evolutionary and ecological hypotheses logistically impossible. A

population is panmictic if every individual has an equal probability of mating with another individual. Disruptions to panmixia result in population subdivision, i.e., allele frequencies will vary between subpopulations with restricted gene flow. The mechanisms that drive population subdivision can occur on both the local scale (e.g., inbreeding or separation of parasites among sympatric host species) and regional or geographic scales, resulting in predictable patterns of genetic structuring (Gorton et al. 2012). Different ecological factors that disrupt panmixia can leave different genetic patterns. By identifying these genetic patterns, one can then indirectly elucidate processes such as mating systems, dispersal patterns or historical population growth and decline (Criscione et al. 2005; Hedrick 2005: Gorton et al. 2012; Nadler 1995). In this dissertation, I will examine the effects of host specificity, host dispersal and the physical characteristics of dendritic river systems on the population structure of *Renifer ancistrodontis* and *Renifer aniarum*. In Chapter II, I will use a co-structure analysis and mitochondrial and microsatellite markers to examine the effects of host specificity on fine-scale population structure of the host specific parasite *R. ancistrodontis* and the generalist *R. aniarum*. The findings of this study do not support the predictions of Nadler's hypothesis regarding the genetic consequences of host specificity but may instead emphasize the important contributions of host dispersal and complex, multi-host life cycles in determining trematode population structure at the local level. In Chapter III, I examine the relative influence of unidirectional stream drift, dendritic ecological networks and host dispersal on *R. aniarum* population connectivity in the Colorado and Concho Rivers in west Central Texas. I found support for Blasco-Costa and colleagues

(2012) prediction that parasites infecting highly motile hosts will not be strongly influenced by unidirectional stream drift. I also determined that *R. aniarum* population structure is intermediate to the population structure expected for autogenic (complete their life cycle within a single habitat type) and allogeneic (complete their life cycle within two or more habitat types) parasites (Criscione et al. 2005).

Parasites are, by definition, inextricably linked to their hosts and hosts are in turn nested within physical habitats subject to human alteration. The Concho water snake, *Nerodia paucimaculata*, is one of the three potential definitive hosts of the parasite *R. aniarum* on the Colorado and Concho Rivers. In Chapter IV, I present the findings of the first three years of the post-delisting genetic monitoring of the Concho water snake, which I conducted concurrently to the research in Chapter III. I determined that the Concho water snake exhibits local population substructure characteristic of a species with high site fidelity and low effective size estimates with evidence of recent bottle necks. I then discuss the implications of the initial population genetic survey and how the results may impact future monitoring efforts.

CHAPTER II

DOES HOST SPECIFICITY SHAPE FINE-SCALE PARASITE POPULATION STRUCTURE? A TEST OF THE PREDICTIONS OF NADLER'S HYPOTHESIS AT THE LOCAL LEVEL

Introduction

Host specificity, the degree to which a parasite exploits different host species, is one aspect of the host-parasite interface that has been implicated as a factor that can affect parasite population structure at both fine and regional scales (Poulin et al. 2011; Nadler 1990; Nadler 1995). Nadler (1995) hypothesized that generalist parasites would exhibit little to no population structure and a higher effective population size, as multiple hosts would facilitate gene flow across the landscape. In contrast host-specific parasites are predicted to exhibit more population genetic structure and smaller effective population sizes. Parasites that are highly host specific infect only a single host species and are dependent on the persistence of that host within a given habitat and movement of that host among habitats. Parasites that use only a single host may be subject to conditions that are both patchy and dynamic over space and time and thus have the potential to experience bottlenecks (or smaller effective population sizes in general) and reduced dispersal potential. Conversely, generalist parasites that use multiple host species are more likely to persist in a habitat if one host is extirpated. These populations will likely not experience dramatic shifts in population size and will likely maintain higher levels of genetic diversity and relatively low levels of among population

substructure. The use of several species of hosts may result in a larger number of host individuals that can support a larger parasite population (and thus larger effective sizes) and facilitate greater dispersal (i.e., gene flow). Variation in host use can thus shape parasite population structure by either restricting gene flow within a single host population or facilitating parasite gene flow in sympatric host populations.

Previous research has found support for Nadler's predictions. Hilburn and Sattler (1986) found no evidence of genetic sub-structure among generalist lone start tick (*Amblyomma americanum)* populations across a broad geographic range. Likewise, Archie and Ezenwa (2011) found little evidence of population structure for a generalist nematode infecting six species of sympatric ungulates. Nonetheless, both Hilburn and Sattler (1986) and Archie and Ezenwa (2011) focused on a single species of parasite and so have no basis of comparison to determine if host-specific parasites exhibit the expected population subdivision. Criscione (2008) proposed that a co-structure analysis, which compares population structure between two or more sympatric species that differ only in their host specificity, would help to elucidate the role of host specificity in determining population substructure. Empirical tests of Nadler's hypothesis using this strategy include four studies.

Falk and Perkins (2013) used mitochondrial markers and tested for isolation by distance to compare the population structure of two species of nematodes, *Parapharyngodon cubensis* and *Spauligodon anolis,* which infect the intestinal tracts of anoles in the Caribbean. *Parapharyngodon cubensis* status as a generalist was based on morphological identifications in literature reports from various squamate hosts

(including non-anoles). *Spauligodon anolis* has only been reported from anoles. Falk and Perkins (2013) found that *P. cubensis* was actually a complex of three different species. Few non-anole hosts were sampled and only one individual *P. cubensis* was found from one non-anole host (Falk and Perkins 2013). The finding that there were multiple species raises the possibility that non-anole hosts may actually harbor additional cryptic lineages of *P. cubensis* and thus calls into question the generalist classification of *P. cubensis*. Also, the authors sampled anoles from across several Caribbean Islands, so it is possible that the variation in parasite population structure may have been due to historical patterns of island colonization. Thus, while Falk and Perkins (2013) found that the most host specific *S. anolis* exhibited greater population structure, there were additional confounding factors that may have affected the observed patterns of diversity and population structure.

Johnson et al. (2002) used a phylogenetic approach to examine the population structure of two genera of lice that infect the wings and body of doves (Columbiformes) using the mitochondrial cytochrome c oxidase subunit 1 (COXI) gene. They determined that members of the more host specific louse genus *Physconelloides* louse exhibited greater structure than the generalist louse genus *Columbicola* in Southern Texas and Northern Mexico (Johnson et al. 2002). However, the species delimitation used by Johnson et al. (2002) was based on previous morphological designations and rather than the molecular data which suggested cryptic species. Moreover, limited sampling over a large geographic area prevented tests for panmixia among host species or localities. Importantly, the observed results may have been a function of differences in dispersal

ability between the parasite genera themselves. The generalist (*Columbicola*) exhibited the least amount of genetic differentiation both among hosts and among localities. It is also known to disperse via phoresy (hitch-hiking) on hippoboscid flies (Johnson et al. 2002). Meanwhile, *Physconelloides* disperse only via direct physical contact between hosts. Thus species-specific dispersal ability may have been responsible for the differences in population structure between *Physconelloides* and *Columbicola*, rather than host specificity.

Mathee et al. (2018) examined the effects of host specificity on population structure and diversity of nest-bound mites in the genus *Laelaps.* The authors to not reference Nadler's hypothesis but refer instead to the specialist-generalist variation hypothesis (SGVH), a hypothesis developed for free-living organisms that has the same genetic predictions for habitat specialists as Nadler's hypothesis predicts for host specific parasites. Both species of mites have direct life cycles, feed briefly from their mouse host and then complete their life cycle within the nest of the host. The more host specific *Laelaps gigantues* infects four species of mice, all in the genus *Rhabdomys*. Each species of *Rhabdomys* is associated with a significantly co-diverged lineage of the host specialist *Laelaps gigantues.* The generalist *L. muricola* infects three species of mice, two from the genus *Mastomys* and one from *Micaelamys* but does not exhibit phylogenetic divergence among hosts. They used cytochrome oxidase subunit 1 (CO1) to determine that the host specific lineages of *L. gigantues* from the two least mobile *Rhabdomys* species exhibited greater population substructure and lower diversity. The two host specific *L. gigantues* lineages from the most mobile *Rhabdomys* exhibited

lower population structure and higher levels of diversity compared to the least mobile hosts but not compared to the generalist *L. muricola*. The generalist *L. muricola* exhibited no population structure and the highest levels of genetic diversity. Thus differences in host dispersal between two host specific lineages also influenced the observed differences in population structure, with greater host dispersal linked to lower population substructure and higher diversity even in host specific lineages. The author's suggest that the SGVH hypothesis (read: Nadler's hypothesis) be modified to apply only to a host specialist that is restricted by limited host dispersal.

While these studies show some support for Nadler's hypothesis, there are some limitations to the systems in which they were tested. Johnson et al. (2002), and Falk and Perkins (2013), used traditional phylogeographical approaches, and had few samples per host species or location to test if they are investigating a true generalist. Similarly both Johnson et al. (2002), Falk and Perkins (2013) were unable to eliminate the possible influences of vicariance, colonization and extinction events that could be influencing structure. Mathee et al. (2018) were able to control for differences in both host specificity and host dispersal but again sampled at a regional scale using phylogeograhic analyses and had small local sample sizes. Mitochondrial markers are well suited to investigations of historical patterns but do not necessarily reflect contemporary ecological processes. Microsatellite markers would be a more appropriate choice of molecular tool to examine contemporary patterns of structure caused by host specificity. For example, Martinu et al. (2018) examined the effects of host specificity on population structure by comparing the population structure of two mtDNA-based lineages of the

sucking louse *Polyplax serrata* that infect mice in the genus *Apodemus.* They used both mitochondrial cytochrome c oxidase subunit 1 (COXI) gene and microsatellite markers to compare genetic diversity, population subdivision and isolation by distance in louse populations across central Europe. They determined that the host specific *P. serrata* lineage B yielded lower genetic diversity, higher F_{ST} values and significant isolation by distance compared to the non-specific *P. serrata* lineage A. Thus they were able to determine that both the mitochondrial and microsatellite data both confirmed the results of Nadler's hypothesis but were still limited by small local sample sizes. Additional work is needed to address the genetic consequences of host specificity at the local-level by testing for panmixia both within and among hosts with larger sampling sizes at fine-scale sampling.

The objective of this study was to test the predictions of Nadler's hypothesis of the effects of host specificity on parasite population structure by conducting a co-structure analysis of fine-scale population genetics between host generalist *R. aniarum* and host specialist *R. ancistrodontis.* This system offers some important advantages compared to previous investigations. The trematodes *R. aniarum* and *R. ancistrodontis* are sympatric parasite species, with similar life-cycles that differ only in their host range, the number of host species used by a parasite. They exhibit complex, three host life cycles involving the potential for both aquatic (snail and tadpole intermediate hosts) and terrestrial (adult frog intermediate host and snake definitive host) transmission. I examined population structure between collection locations and among hosts (i.e., infrapopulation a subpopulation of parasites infecting an individual host, compare

genetic diversity, and estimate effective population size for the two species. Nadler's hypothesis predicts that the generalist *R. aniarum* will have lower population structure (lower F_{ST} values) between collection locations and among hosts due to the increased dispersal potential of using multiple host species to facilitate gene flow. *R. aniarum* is also predicted to have higher genetic diversity and effective population size due to a larger number of potential hosts drawn from multiple species of definitive host which can support larger parasite population sizes and reduce the probability of extirpation. Conversely the specialist *R. ancistrodontis* is predicted to have greater among host structure due to more limited dispersal potential from infecting a single definitive host species. *R. ancistrodontis* is also predicted to exhibit lower genetic diversity and a lower effective population size. As host-specificity is thought to be a driving force in parasite diversification, identifying host specificity as a cause of local-scale structure for parasites with complex life cycles and semiaquatic transmission will prove a significant contribution in support of this macroevolutionary prediction. Additionally, by focusing on a local-scale, this study will reduce confounding historical and geographical factors.

Methods

Study System

Renifer aniarum and *Renifer ancistrodontis* are members of the family Plagiorchidea, subfamily Reniferinae (Byrd 1935; Tkach 2008). Species in the Genus *Renifer* have a 3-host life cycle, where freshwater snails are first intermediate hosts, tadpoles are second intermediate hosts and snakes are definitive hosts (Byrd 1935; Talbot 1933; Talbot 1934; Tkach 2008). Eggs are released from adult worms and voided

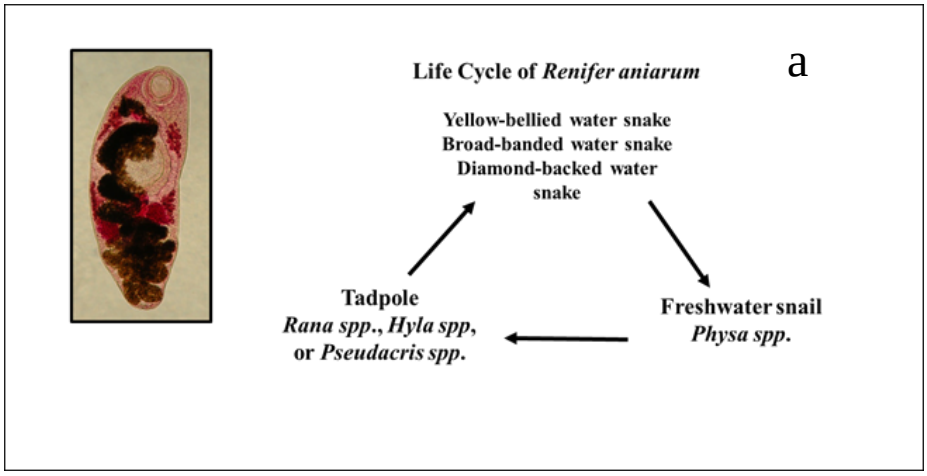
with the host feces (Figure 1.2). Eggs are consumed by an aquatic snail. Experimental infections and extensive collections suggest that the parasite is infective only to pulmonate snails in the genus *Physa* (Byrd 1935; Santoro et al. 2011). Members of the anuran genera *Lithobates, Hyla, Pseudacris, Lithobates,* and *Anaxyrus* have all been demonstrated to serve as intermediate hosts (Walker 1937; Sears et al. 2012). Byrd (1935) observed that the encysted metacercaria from the tail of the tadpole appear to be retained in the body of the adult frog although it was difficult to determine if these represented previously undetected individuals already present (Byrd 1935). Tadpoles are trophically transmitted to the snake definitive host, where parasite sexual reproduction occurs. Immature worms appear in the mouth approximately 25 days after the initial infection and are gravid after 35 days. Transmission from consuming infected adult frogs has not been confirmed.

Renifer aniarum has previously been reported from 24 species of snake in the continental US and one species in Italy where it has been introduced (McAllister and Bursey 2008: Santoro et al. 2011). While other members of the genus *Renifer* co-infect these snakes, *R. aniarum* is easily distinguishable from other members of the genus by the location of the genital pore, which is even with the oral sucker, and the pre- and post-acetabular clusters of vitelaria follicles (Dryer and Ballard 1991). Unlike the generalist *R. aniarum, R. ancistrodontis* is thought to exhibit host specificity in the definitive host based on collections rather than life cycle studies. *Renifer. ancistrodontus* has only been collected from eastern and western cottonmouths (*Agkistrodon. piscivorous spp*.) even

where multiple aquatic snakes occur sympatrically (McAllister et al. 2008; Detterline et al. 1984, Janecka, this study).

Survey Site and Field Surveys

Surveys were conducted at Gus Engeling (GEWMA) and Richland Creek Wildlife (RCWMA) Wildlife Management Areas in Anderson, Freestone and Navarro counties in east-central Texas in the Middle Trinity River Ecosystem Project. They are separated at their nearest point by approximately 10.4 km of privately owned properties. During the spring of 2012, watersnakes and cottonmouths were collected using minnow traps, active search and nocturnal road surveys (Table 1.2). Snakes were processed on site using non-lethal methods and then released (IACUC 2015-0030). Collection locality information and morphometric data was collected from each individual and the snakes were individually marked with a scale clip number. Tissue from scale clips was stored in 70% ethanol. All flukes within the oral cavity and upper ½ inch of the esophagus were removed, heat-killed and stored in 70% ethanol for genetic analysis. *Agkistrodon piscivorus* were anesthetized with a sub-lethal 50% MS-222 injection into the coelom of the snake prior to handling following approved protocols.



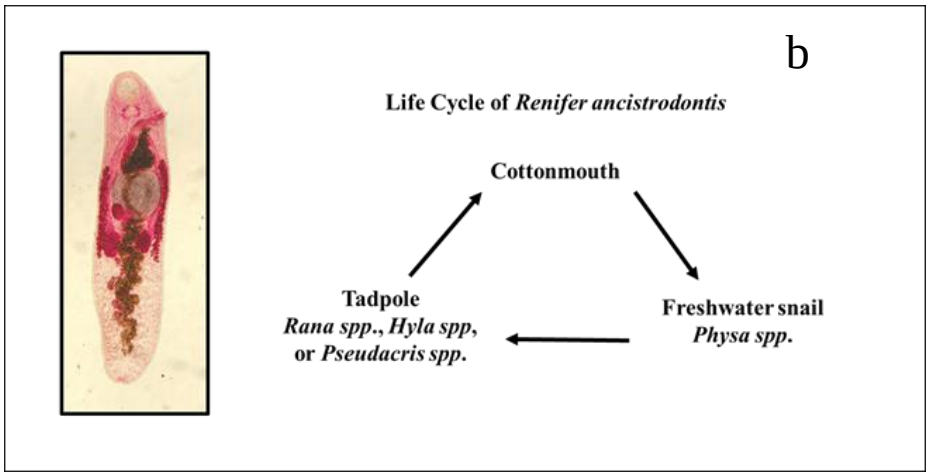


Figure 1.2. a. Life cycles of the generalist parasite *R. aniarum* and host-specific parasite *R. ancistrodontis* (**b**). The adult flukes of *R. aniarum* infect the mouths of three *Nerodia* species at Gus Engeling and Richland Creek Wildlife Management Areas. The life cycle of *R. ancistrodontis* differs in that only uses cottonmouths (*Agkistrodon piscivorus*) as its definitive host.

Table 1.2. Mean parasite prevalence and intensity of infection for *R. aniarum* and *R. ancistrodontis* collected from Gus Engeling (GEMA) and Richland Creek (RCWMA) Wildlife Management Areas; Texas, USA in 2012.

Host Species	N		Parasite Infection	Prevalence		Mean Intensity ±SE	
	GEWMA	RCWMA		GEWMA	RCWMA	GEWMA	RCWMA
Nerodia erythrogaster	6	7	*R. aniarum*	0.5	0.28	16.33±5.51	15.5±4.56
N. fasciata	4	4	*R. aniarum*	1	0.5	10.5±5.51	1.5±0.478
N. rhombifer	7	11	*R. aniarum*	0.14	0.45	1±0.142	7±1.91
Agkistrodon piscivorus	4	10	*R. ancistrodontis*	0	0.9	0	15.33±4.37

Microsatellite Development, Genotyping and Analysis

Three pooled specimens from both *R. aniarum* and *R. ancistrodontis* collected in 2011 were submitted to the Sequencing and Genotyping Facility at the Cornell Life Sciences Core Laboratory Center (Ithaca, NY) in order to develop microsatellite libraries as described in the methods of Nali et al. (2014). This resulted in libraries of 2,092 and 510 potential microsatellite loci for *R. aniarum* and R. *ancistrodontis* respectively.

A total of 87 and 119 potential loci were screened for 8 *R. aniarum* and 16 *R. ancistrodontus* individuals respectively. Cross-species amplification was attempted between the two species but all markers amplified only in the species for which they were designed. This process resulted in 12 informative and scorable loci for *R. aniarum* (Table 2.2) and 9 loci for *R. ancistrodontis* (Table 3.2). The M13 method was used for genotyping and screening by attaching a 20-bp tag to the 5' end of the forward primer (TGTAAAACGACGGCCA) (Schulke 2000). A short tail sequence (GTTTCT) was added to the 5'end of the reverse primer to reduce polyadenylation (Detwiler and Criscione 2011). PCR amplification was performed in 10 µL reactions containing 3.1 µL ultrapure water, 5 µl 2X Qiagen Type-IT kit Master Mix, 0.16 µL fluorescent-labeled M13 primer (Applied Biosystems: FAM), 0.08 µL M13-labeled forward primer, 0.16 µL of 10 µM reverse primer and 1.5 µL of genomic DNA. The thermocycler profile was 94 ºC for 5 minutes, 31 cycles of 94ºC for 30 seconds, 56º C for 45 seconds, 65º C for 45 seconds, followed by nine cycles of 94ºC for 30 seconds, 53ºC for 45 seconds 65 ºC for 45 seconds and extension at 65 ºC for 10 minutes. PCR-product was visualized on a 2% agarose gel run in 0.5X TBE buffer at 95 V for 45 minutes. Reactions that yielded

discrete bands in the expected product size range were sent to the DNA Analysis Facility on Science Hill at Yale University (New Haven, CT, USA) and visualized on a 3730xl 96-capilary Genetic Analyzer with 500-LIZ size standard. Samples were genotyped on GeneMarker 2.6.4.

Identification of Clones

Multilocus genotypes (MLGs) were assessed using GENALEX 6.502 in order to identify potential clonemates (genetically identical individuals) that may be present due to asexual reproduction in the snail host. Given the high diversity of the loci it is extremely unlikely that non-clonemates would have identical multilocus genotypes. One representative of each clone was included in the subsequent analyses to prevent artificial inflation of F_{ST} due to clonemates (Criscione et al. 2011).

Genetic Diversity, F_{IS}, and Mating System

Genotypic disequilibrium for pairs of microsatellite loci was tested using GENEPOP web versions 4.2 (Raymond and Rousset 1995). Statistical significance was determine using the log likelihood ratio statistic with Markov chain parameters all set for 5000 dememorizations, batches and iterations per batch. Gene diversity (H_S), the number of alleles per locus (A_N), and allelic richness (A_R) (rarefied number to the lowest region sample size of N = 57) were calculated in FSTAT 2.39 (Goudet 2001). Statistical tests for differences in average genetic diversity indices and F_{IS} values between species were examined using the non-parametric Kruskal-Wallis test and implemented in R using the heirfstat, dbplyr and coin libraries (R Core Development Team 2017). Significance values were examined using the permutation tests implemented in the coin

library for non-normally distributed data. Post-hoc Mann-Whitney U-tests were implemented in dbpylr with Bonferroni corrections for multiple comparisons. Estimates of F_{IS} (which quantifies the proportional change in heterozygosity due to deviations in Hardy Weinberg equilibrium, HWE) and tests of whether F_{IS} deviated from 0 (i.e. expectations under HWE) were conducted in in FSTAT 2.39 at 999 randomizations of alleles among individuals (Goudet 2001). Elevated F_{IS} values were observed in the data set upon preliminary analysis. For this reason, identity disequilibrium was then examined using the program RMES set to 1000 iterations of sampling. Testing for the presence of identity disequilibrium can help to determine if inflations in F_{IS} are due to inbreeding (which could result from self-mating) or are artificially inflated due to the presence of null alleles in the data set. Subsequently, the program INEst 2.2 (Chybicki and Burczyk 2009) was used to obtain multilocus estimates of the inbreeding coefficients for each species and population and to determine if each population fit the model associated with the presence of inbreeding (via self-mating in hermaphrodites) or with the presence of null alleles. INEst jointly estimates inbreeding and null alleles and then provides a means to test if either or both are needed to explain the observed patterns. The program was implemented with 500,000 cycles and the model with the lowest DIC criteria was selected as the best fit for the data set. Finally the mating systems were examined by estimating the selfing rate in COLONY v 2.0.6.4 (Jones and Wang, 2010) using two different sets of parameters. COLONY was selected because this program incorporates estimates of genotypes errors such as can occur when null alleles are present and also incorporates estimates of mating system and effective size.

Table 2.2. Informative microsatellite loci developed for *R. aniarum*.

Primer Name	Primer Sequence (5' to 3')	Repeat Motif	T_a(°C)	Size Range (bp)
ReAn 627	F: TGTAAAACGACGGCCAGT	AGC	56	347-426
	R: GAGACTTCAAGCCTCGAACTG			
ReAn 1082	F: GGCTAACCCAGTTTCGAAGC	ACT	56	244-378
	R: TGAGTCGGCATTAGTTGATTGG			
ReAn 5773	F: ACTGCCAACACTCGATTGATC	ACAG	56	170-304
	R: ACGTACAATAAATGCAGCGC			
ReAn 8483	F: TTCAACCACACTTCGTTCGC	AC	56	155-193
	R: AGTTCAACTGATCAACGACGAG			
ReAn 3858	F: GGAGCTTGCCACAACCAC	AAC	56	155-260
	R: GACTCGGCGTTCTAAAGTGC			
ReAn 2341	F: ACCCATGCAACCAAGATGAG	AG	56	380-406
	R: GGTACTCTCCTGGGATCGTG			
ReAn 5592	F: TGACCGCAGATTTGACCAATG	ATC	56	298-366
	R: GGTCTACGTGCTTGTGTTCG			
ReAn 990	F: ACCCGCTTGTCATATTCAGTG	AAC	56	169-202
	R: TGAACCCACAATTTCGCTGG			
ReAn 1248	F: TCGGAGAACAAACCACCAC	AAG	56	395-464
	R: AACTTGAAGCAAAGGTGGCC			
ReAn 2450	F: CTAAAGCGTCTCGTTCCTTGATTC	ACAT	56	135-173
	R: GTCACAATGGAGTTTCAAATGTCG			
ReAn 1065	F: AGTGCCTGTTCGCTGATTTC	AAG	56	161-215
	R: CTGTGGAAAGTCGTTGGTATTG			
ReAn 5219	F: CTGATGGCGATATTACTTCCGC	ACT	56	243-333
	R: GGACCAACAGATTATATGTCAGTACG			

Table 3.2. Informative microsatellite loci developed for *R. ancistrodontis*.

Primer Name	Primer Sequence (5' to 3')	Repeat Motif	T_a(°C)	Size Range (bp)
ReAg 1452	F: GCATCATCAGCATGCGTAGG	AC	56	209 -227
	R: CAGCGTGGCGTGATAATAAAC			
ReAg 1505	F: TGTCATCACGTTTGTCAACCG	AG	56	168 -270
	R: CATGCTCTTTGGTCTCCGC			
ReAg 1520	F: ACCCAACCACTATGATCACAC	AC	56	206 - 252
	R: CCAAATGTGTCTTCTTGCTCAG			
ReAg 424	F: GCACCAAATCTACCTCGCTC	ACAG	56	149 - 204
	R: GCTGTGCCCAAGAGCAGG			
ReAg 757	F: TTAACACCACCGCCCTAGTC	ATC	56	172-234
	R: GGCACAGTCGAAGTCAGTTTC			
ReAg 1095	F: GGAAACAGTCGAAACAAAGCG	AAAC	56	280-344
	R: AATGATGGAAAGAGCGACCG			
ReAg 97	F: GGCAACATCATCAGCGGTAC	AAC	56	152 - 212
	R: ATGAGAATTGCGTTAACACCTG			
ReAg 287	F: TCCGGAGTGACATAATCTTGTG	AAC	56	143-202
	R: TTGAGTTGCCGCATTGTCTG			
ReAg 787	F: TGTGACAACTTTCGGCTTCG	ATCC	56	190 - 366
	R:CTAGTGGAAGCCGAGACTCC			

The first parameter set included male and female polygamy with inbreeding for a monoecious organism, length of run was set to very long under the full-likelihood method with very high precision, three runs, allele frequencies estimated and then updated from the dataset. Sibship scaling was set to yes and no sibship prior was assumed. Allelic dropout rate was set to 0.01 and false allele error rate was set to 0.005. COLONY was then run again using the same set of parameters with the exception of the allelic dropout rates. Estimates of allelic dropout rates for each marker in each species and each population were obtained from the results in INEst 2.2. These values were substituted for the initial allelic dropout rate of 0.01. All other parameter setting were kept the same.

Estimates of Population Structure

Estimates of population structure were performed between sampling locations and among host species for *R. aniarum,* and among infrapopulations within individual snakes for both *Renifer* species. An overall test of genetic differentiation (and estimation of F_{ST}) along with pairwise tests of genetic differentiation (and pairwise estimates of F_{ST}) were conducted in Arlequin 3.5.2.2 (Excoffier et al. 2010). The statistical significance of population differentiation was implemented in heirfstat (Goudet 2005).

Due to the potential presence of null alleles within the data set, estimates were also conducted in FreNA (Chapuis and Estoup 2007) which estimates null allele frequencies for each locus and populations and estimates an unbiased F_{ST} values. The program hierfstat was implemented in R to examine the potential effects of hierarchical

structure between collection locations and among infrapopulations. Randomization testing was implemented at 15,000 permutations. I then used the Bayesian clustering model based clustering implemented in STRUCTURE 2.3.4 (Pritchard et al. 2000), which partitions individuals based on HWE and linkage equilibrium. The input parameters for STRUCTURE were correlated allele frequencies, and the admixture model. STRUCTURE was run with 5,000,000 iterations with a burn-in of 500,000 iterations for *K* (i.e., the number of possible clusters) values 1 to 10 with 10 replications of each possible *K* value. STRUCTURE was used to examine population differentiation among hosts for both species and among locations for *R. aniarum*.

Estimates of Effective Population Size

The effective population size (N_e) was estimated in two ways. First, I used the linkage disequilibrium estimator that was devised by Waples (2006) and is implemented in the software NEestimator (Waples and Do 2008). However, the bias correction implemented in the program NEestimator was based on theory developed for dioecious rather than monoecious species and may not be applicable when self-mating occurs within the system. For this reason, the program COLONY v 2.0.6.4 (Jones and Wang, 2010) was also used to estimate the effective population size. This program estimates the effective population based on the frequency of full-siblings and half siblings present in the sample. COLONY was run twice for each species and for each collection site (GEWMA and RCWMA) for *R. aniarum*, using two different parameter sets as described above for mating system determination.

Mitochondrial Sequencing and Analysis

No microsatellite markers cross-amplified between *R. aniarum* and *R. ancistrodontis*. In order to make a direct comparison of genetic diversity between the two species and to test for reciprocal monophyly, I sequenced the mitochondrial DNA cytochrome oxidase subunit 1 (CO1) gene (~940 bp) of a subset of the genotyped individuals within the sample set (*R. aniarum:* RCWMA, N=20, GEWMA, N=16; *R. ancistrodontis* : RCWMA, N=33). Forward primer was JB3 (5' - TTTTTTGGGCATCCTGAGGTTTAT-3') (Bowles et al. 1993) and reverse primer was CO1-R trema (5'AACAAATCATGATGCAAAA GG -3') (Muira et al. 2005).

PCR amplification was performed in 20 µL reactions containing 3.2 µL of ultrapure water, 10 µL of Amplitaq Gold 2x Master Mix, 0.4 µL of 20 mM of CO1 forward primer, 0.4 µL of 20 mM of CO1 reverse primer, and 6 µL of DNA template. Thermocycler profile was 95 ºC for 10 minutes, 45 cycles of 95 ºC for 30 seconds, 45 ºC for 30 seconds, 72º C for1 minute, and extension at 72 ºC for 7 minutes. PCR-product was visualized on a 2% agarose gel run in 0.5X TBE buffer at 95 ºC for 45 minutes. PCR products were purified with the Ultra Clean PCR clean-up Kit (MO BIO Laboratories, Inc., Solana Beach, California) and sent to the DNA Analysis Facility on Science Hill at Yale University (New Haven CT, USA) for sequencing. Consensus sequences of reads in both the forward and reverse directions were assembled and edited manually in Sequencher 3.0 (Gene Codes Corporation, Ann Arbor, Michigan, U.S.). Sequences were aligned and three mitochondrial DNA phylogenies were constructed in MEGA-X 10.0.5 (Kumar et al. 2018): one for both species together and two for each

species separately. Phylogenies were constructed using the maximum likelihood method and best fit model selection. The most appropriate model selected for the phylogeny containing both species was the HKY+G, and the model selected for the phylogenies for each species alone was HKY. Measures of diversity for the COI sequence data, including number of variable sites (V_S), average number of nucleotide differences, number of haplotypes (N_{HAP}), haplotype diversity (D_{HAP}), and nucleotide diversity (Theta) were calculated in DNASP 4.10.8 (Nei and Li 1979; Rozas and Rozas 1999). An AMOVA implemented in DNASP was used to examine population differentiation between collection locations for *R. aniarum* and estimates of significance were obtained from 1000 permutations.

Results

Identification of Clones

Twelve microsatellite markers were amplified in 161 *R. aniarum* individuals (GEWMA: N=92; RCWMA: N=69). Nine microsatellite markers were amplified in 138 individuals all from RCWMA for *R. ancistrodontis*. The identification of clonemates based on identical multilocus genotypes was carried out in GENALEX 6.502. A total of 8 clones had clonemates and all other clones were represented by a single individual *R. aniarum* data set; 5 from infections in RCWMA and three from GEWMA (Table 4.2). Groups of clonemates ranged in size from a pair to four individuals. Similarly, 6 clonemates groups were identified in *R. ancistrodontis* and ranged in size from two to five individuals (Table 5.2). Clonemates were always located within the same individual snake host, indicative of clumped transmission after release from the first intermediate

host. A single representative individual from each clone was retained in the data to reflect the prior adult generations mating systems and the other individuals were removed. The proportion of clonemates to unique clone genotypes was 0.89 for *R. aniarum* and 0.92 for *R ancistrodontis* The removal of clones resulted in a reduced data set of 144 *R. aniarum* individuals (GEWMA: N=87; RCWMA: N=57) and 127 *R. ancistrodontis* individuals. All subsequent analyses described below are carried out using the reduced dataset.

Genetic Diversity, F_{IS}, and Mating System

Nine of the twelve loci for *R. aniarum* showed significant deviations from HWE in GEWMA and RCWMA (Table 6.2). With the 12 loci, there were 66 pairwise comparisons for tests of linkage disequilibrium. At a nominal alpha level of 0.05, one would expect 3.3 comparisons to be significant by chance alone. Fifteen pairs of loci were in linkage disequilibrium from GEWMA and 21 pairs of loci were significant for RCWMA. Thus, there was global significant linkage disequilibrium for *R. aniarum* in both GEWMA and RCWMA locations (exact binomial $p<0.001$ for both locations). For *R. ancistrodontis*, there was also linkage disequilibrium between pairs of loci. There are 36 pairwise comparisons for 9 loci. At a nominal level of 0.05, one would expect 1.8 loci to be in LD by chance alone and 8 pairwise comparisons were significant (exact binomial $p<0.001$). Linkage disequilibrium can be caused by the presence of cryptic population structure (the Wahlund effect) within the population structure. Although non-random mating by itself does not cause LD, it is not uncommon to find LD in hermaphroditic species where bottleneck or founder events may be common (e.g., a

single individual can found a new population). Estimates of microsatellite diversity for *R. aniarum* (GEWMA and RCWMA) and *R. ancistrodontis* are given in Table 6.2 and Table 7.2. Overall levels of genetic diversity were high for both *Renifer* species and for both collection sites (Table 6.2 and 7.2). Allelic richness ranged from 8.49 to 29.388 for *R. aniarum* from GEWMA to 7.96 to 27.77 in RCWMA. Allelic richness was similarly high for *R. ancistrodontis*, with values from 8.995 to 25.82. There was no statically significant difference between allelic richness among the three groups ($X^2_{(2)}$=0.251, p=0.8819). Mean gene diversity (H_S) was high for *R. aniarum* in both GEWMA (0.944) and RCWMA (0.835). Mean gene diversity was similarly high in *R. ancistrodontis* (0.826). There was no statistically significant difference in gene diversity among *R. aniarum* from RCWMA and GEWMA and *R. ancistrodontis* ($X^2_{(2)}$=1.3002, p=0.522). F_{IS} values for *R. aniarum* ranged from -0.040 to 0.202 for GEWMA with a multilocus F_{IS} value of 0.164. F_{IS} values from RCWMA ranged from -0.027 - 0.296 with a multilocus F_{IS} value of 0.082. F_{IS} values for *R. ancistrodontus* ranged from 0.079 to 0.641 with a multilocus F_{IS} of 0.465 (Table 7.2). F_{IS} values were significantly different among the three groups ($X^2_{(2)}$=14.595, p=<0.001). A *post hoc* pairwise Wilcox test indicated that F_{IS} values for *R. ancistrodontis* (*R. ancistrodontis*- *R. aniarum* GEWMA, p=0.007; *R. ancistrodontis*- *R. aniarum* RCWMA, p=0.009) were significantly different but were not significantly different between *R. aniarum* from GEWMA and RCWMA (p=0.266). Elevated F_{IS} values can potentially be caused by the presence of null alleles in the dataset, inbreeding or the Wahlund effect. The results of the identity disequilibrium (ID) test implemented in RMES for *R. aniarum* $\hat{g}_2$ was not significantly

greater than 0 for GEWMA (p=0.639) or RCWMA (p=0.795). $\hat{g}_2$ for *R. ancistrodontus* was marginally non-significant (p=0.056). This suggests that nonrandom mating i.e. inbreeding/selfing may be responsible for the higher F_{IS} in *R. ancistrodontis*. Though, null alleles may be present in the data sets for both species as the ID results were not concordant with the observed levels of F_{IS}.

Model testing in INEst was used to determine the degree to which null alleles and inbreeding could be affecting the data set. I compared the models NFB (which includes inbreeding and null alleles) and NB (which excludes inbreeding but includes null alleles). The NFB model had the lowest DIC_1 value for each species and population, indicating that inbreeding was most likely occurring in each population (Table 8.2). The average inbreeding coefficient obtained from INEst was highest for *R. ancistrodontis* (F=0.2355). Average inbreeding coefficient obtained from INEst was low for both populations of *R. aniarum* (GEMWA F=0.08: RCWMA F=0.04) but was still estimated to be the most likely model for this species, contrary to the results suggested by the ID. The inbreeding coefficient estimated in COLONY incorporates error rates estimated by the program, which accounts for some of the variation in these values between programs. Inbreeding and selfing rates were highest for *R. ancistrodontis* under both parameter sets in COLONY and lower for *R aniarum* from both collection locations. Higher rates of inbreeding in *R. ancistrodontis* are likely to be driving the significant differences in F_{IS} observed between the two species.

Table 4.2. Clonemates identified by GENALEX for *R. aniarum*.

Individual ID	Clonal Group	Collection Site	Included in Reduced Data Set
Es007By12w004	**1**	**RCWMA**	
Es007By12w010	**1**	**RCWMA**	**X**
Es007By12w005	**1**	**RCWMA**	
Es007By12w004	**1**	**RCWMA**	
Es007By12w018	**2**	**RCWMA**	
Es007By12w008	**2**	**RCWMA**	**X**
Es007By12w001a	**2**	**RCWMA**	
Rs014y12w021	**3**	**RCWMA**	
Rs014y12w004	**3**	**RCWMA**	**X**
Rs014y12w008	**3**	**RCWMA**	
Rs014y12w004	**3**	**RCWMA**	
Es007By12w021	**4**	**RCWMA**	
Es007By12w014	**4**	**RCWMA**	**X**
Rs014y12w010	**5**	**RCWMA**	
Rs014y12w006	**5**	**RCWMA**	**X**
Rs014y12w007	**5**	**RCWMA**	
Es002By12w033	**6**	**GEWMA**	**X**
Es002By12w020	**6**	**GEWMA**	
Fs008y12w011	**7**	**GEWMA**	**X**
Fs008y12w012	**7**	**GEWMA**	
Fs008y12w005	**7**	**GEWMA**	
Fs008y12w003	**7**	**GEWMA**	
Es002By12w003	**8**	**GEWMA**	**X**
Es002By12w017	**8**	**GEWMA**	

Table 5.2. Clonemates identified by GENALEX for *R. ancistrodontis*.

Individual ID	Clonal Group	Collection Site	Included in Reduced Data Set
As10y12w001	**1**	**RCWMA**	**X**
As10y12w003	**1**	**RCWMA**	
As13y12w015	**2**	**RCWMA**	
As13y12w013	**2**	**RCWMA**	**X**
As13y12w007	**2**	**RCWMA**	
As16y12w014	**3**	**RCWMA**	**X**
As16y12w028	**3**	**RCWMA**	
As11y12w009	**4**	**RCWMA**	
As11y12w018	**4**	**RCWMA**	**X**
As18y12w002	**5**	**RCWMA**	
As18y12w012	**5**	**RCWMA**	
As18y12w005	**5**	**RCWMA**	
As18y12w010	**5**	**RCWMA**	**X**
As18y12w004	**5**	**RCWMA**	
As17y12w010	**6**	**RCWMA**	**X**
As17y12w004	**6**	**RCWMA**	
As17y12w009	**6**	**RCWMA**	

Table 6.2. Measures of microsatellite diversity and tests of HWE for *R. aniarum* from Gus Engeling Wildlife Management Area 2012 **(**N= 87). Clonemates were removed from the dataset.

Locus	**N**[a]	A_N[b]	A_R[c]	H_O[d]	H_S[e]	F_{IS}[f]
ReAn 627	87	14	12.972	0.621	0.859	**0.277**
ReAn 1082	87	33	29.388	0.851	0.954	**0.108**
ReAn 5773	87	20	18.783	0.598	0.929	**0.357**
ReAn 8483	87	12	11.028	0.471	0.523	**0.099**
ReAn 3858	87	20	18.458	0.724	0.908	**0.202**
ReAn 2341	87	11	10.315	0.678	0.790	**0.141**
ReAn 5592	87	9	8.496	0.724	0.783	0.076
ReAn 990	87	25	22.532	0.747	0.900	**0.170**
ReAn 1248	87	26	24.227	0.770	0.947	**0.186**
ReAn 2450	87	22	20.003	0.874	0.926	0.056
ReAn 1065	86	10	9.247	0.756	0.727	-0.040
ReAn 5219	87	27	24.905	0.701	0.944	**0.257**
Mean/Multilocus F_{IS}		19	17.529	0.709	0.944	**0.164**

[a] Number of individuals genotyped

[b] The number of alleles per locus

[c] Allelic richness

[d] Observed heterozygosity

[e] Gene diversity

[f] Bold F_{IS} values indicate significant deviations from HWE ($p < 0.05$)

Table 7.2. Measures of microsatellite diversity and tests of HWE for *R. aniarum* from Richland Creek Wildlife Management Area 2012 (N= 57). Clones were removed from the dataset.

Locus	**N**[a]	A_N[b]	A_R[c]	H_O[d]	H_S[e]	F_{IS}[f]
ReAn 627	57	9	8.929	0.684	0.838	**0.183**
ReAn 1082	57	27	26.819	0.825	0.951	**0.132**
ReAn 5773	57	19	18.927	0.754	0.903	**0.164**
ReAn 8483	57	11	10.890	0.509	0.521	0.024
ReAn 3858	57	18	17.930	0.912	0.888	-0.028
ReAn 2341	57	10	9.964	0.789	0.769	-0.027
ReAn 5592	57	8	7.964	0.825	0.796	-0.036
ReAn 990	57	28	27.7785	0.649	0.922	**0.296**
ReAn 1248	57	25	24.788	0.860	0.942	**0.088**
ReAn 2450	57	19	18.823	0.895	0.901	**0.007**
ReAn 1065	57	10	9.928	0.684	0.654	**-0.046**
ReAn 5219	55	23	23.000	0.8318	0.945	**0.135**
Mean/Multilocus F_{IS}		23	23	0.835	0.835	0.082

[a] Number of individuals genotyped

[b] The number of alleles per locus

[c] Allelic richness

[d] Observed heterozygosity

[e] Gene diversity

[f] Bold F_{IS} values indicate significant deviations from HWE ($p < 0.05$)

Table 8.2. Results from the analysis of inbreeding and null allele models in INEst 2.2. The model with the lowest DIC value is selected as being the most appropriate for the data set in question.

Species	Model	DIC_1 Value	Average Inbreeding Coefficient
R. aniarum-GEWMA	NFB	**16563.507**	0.0884
	NB	16692.345	
R. aniarum-RCWMA	NFB	**10355.784**	0.0476
	NB	10411.983	
R. ancistrodontis	NFB	**15744.954**	0.2355
	NB	16189.307	

Population Structure

I first examined *R. aniarum* population structure between GEWMA and RCWMA. I estimated F_{ST} between GEWMA and RCWMA with and without correction for null alleles. F_{ST} values for the host generalist *R. aniarum* individuals collected from GEWMA and RCWMA were low (0.0059) but were significantly different (p=0.002). Results from the analyses with and without accounting for null alleles were nearly identical (Table 9.2) suggesting that while technical artifacts may be present within the *R. aniarum* dataset, the observed patterns of genetic diversity and population subdivision are still likely to be biologically meaningful. I then examined *R. aniarum* population structure between GEWMA and RCWMA corrected for potential infrapopulation structure among hosts with a hierarchical analysis implemented in heirfstat. This resulted in a hierarchical F_{RT} value of 0.02 ($p<0.001$) p between GEWMA and RCWMA. The results of the Bayesian clustering analysis implemented in STRUCTURE indicated genetic population clusters were not associated with the collection location (Figure 2.1).

I then examined infrapopulation structure among hosts for *R. aniarum*. Pairwise F_{ST} values among hosts for *R. aniarum* are given in Table 10.2. F_{ST} values among host individuals were similarly low, ranging from 0.011 to 0.095. Global F_{ST} values among host were also low but significant among infrapopulations populations (F_{ST}=0.026, p=0.001). Global F_{ST} values with and without the correction for the presence of null alleles were again almost identical (Table 11.2). The results of the STRUCTURE analysis among hosts for *R. aniarum* are shown in Figure 2.1. Multiple genetic clusters

were identified but were not strongly associated with individuals except for two individuals, *N. erythorogaster* 2B from GEWMA and *N. rhombifer* 18 from RCWMA. Population substructure for parasites from these individuals was visible through K=5. However, admixture between genetic clusters was visible within the two individuals.

Finally, I examined infrapopulation structure for *R. ancistrodontus.* Pairwise estimates of among host population structure for *R. ancistrodontis* are given in Table 12.2. F_{ST} values among hosts exhibited more variation in range (0.00-0.124). Pairwise F_{ST} values were significant between some but not all hosts. Pairwise F_{ST} values for the infrapopulation within individual *A. piscivorus* number 10 exhibited moderate levels of population substructure while all other individuals exhibited low population substructure. Global F_{ST} values for infrapopulation subdivision was low but significant (F_{ST}= 0.014, p=0.001). Global F_{ST} estimates for *R. ancistrodontis* infrapopulation substructure increased from 0.0139 to 0.021 when corrected for the presence of null alleles in the data set (Table 13.2). No visible infrapopulation structure was detected in the STRUCTURE analysis (Figure 3.2).

Table 9.2. Results of F_{ST}^{A} estimates between GEWMA and RCWMA areas for *R. aniarum*. Corrected F_{ST}^{N} values are based on estimates from the program FreNA which re-estimates F_{ST} values based on the estimated rates of null alleles within the data set. Significance values given above the diagonal using the Weir and Cockerham theta estimate (1984), 999 permutations.

	GEWMA-RCWMA	*P* value
F_{ST}^{A}	0.0059	0.002
F_{ST}^{N}	0.006578	0.002

Table 10.2. Pairwise measures of F_{ST} based on 12 microsatellite markers among hosts infected with more than a single worm (N=140). Host NeFa5, NeRh 9, NeRh16, and NeRh25 were each infected with a single *R. aniarum* individual and were excluded from the analysis. Significance values given above the diagonal using the Weir and Cockerham theta estimate (1984), 10,000 permutations.

	NeEr2A	**NeEr2B**	**NeEr3**	**NeEr4**	**NeEr7B**	**NeFa3**	**NeFa6**	**NeFa8**	**NeFa9**	**NeFa10**	**NeRh11**	**NeRh14**	**NeRh18**
NeEr2A		0.0005	0.1071	0.0019	0.0006	0.1273	0.2079	0.0107	0.0678	0.0518	0.0141	0.0023	0.1716
NeEr2B	0.0625		0.0007	0.0001	0.0001	0.0007	0.2798	0.0004	0.0002	0.0122	0.0073	0.0001	0.0152
NeEr3	0.0205	0.0209		0.0007	0.0002	0.2876	0.2195	0.1651	0.0104	0.0411	0.0130	0.0009	0.0158
NeEr4	0.0588	0.0271	0.0278		0.0003	0.0003	0.1489	0.0046	0.0342	0.0513	0.0320	0.0001	0.1096
NeEr7B	0.0591	0.0283	0.0257	0.0282		0.0004	0.2858	0.0045	0.0148	0.1122	0.0055	0.0001	0.0131
NeFa3	0.0307	0.0408	0.0054	0.0521	0.0420		0.2461	0.0447	0.1582	0.0711	0.0582	0.0030	0.0015
NeFa6	0.0418	0.0099	0.0149	0.0221	0.0102	0.0197		0.4528	0.4732	0.3455	0.2398	0.1715	0.1848
NeFa8	0.0370	0.0126	0.0046	0.0170	0.0125	0.0159	0.0000		0.0546	0.4683	0.0637	0.0001	0.0127
NeFa9	0.0386	0.0384	0.0247	0.0205	0.0198	0.0155	0.0000	0.0133		0.4216	0.0069	0.0114	0.0109
NeFa10	0.0517	0.0296	0.0226	0.0225	0.0140	0.0300	0.0111	0.0000	0.0020		0.0081	0.0024	0.0657
NeRh11	0.0698	0.0318	0.0298	0.0260	0.0368	0.0313	0.0212	0.0177	0.0484	0.0590		0.0008	0.3164
NeRh14	0.0609	0.0392	0.0240	0.0378	0.0438	0.0361	0.0224	0.0267	0.0266	0.0437	0.0521		0.0004
NeRh18	0.0224	0.0191	0.0232	0.0134	0.0220	0.0528	0.0276	0.0203	0.0399	0.0297	0.0074	0.0428	

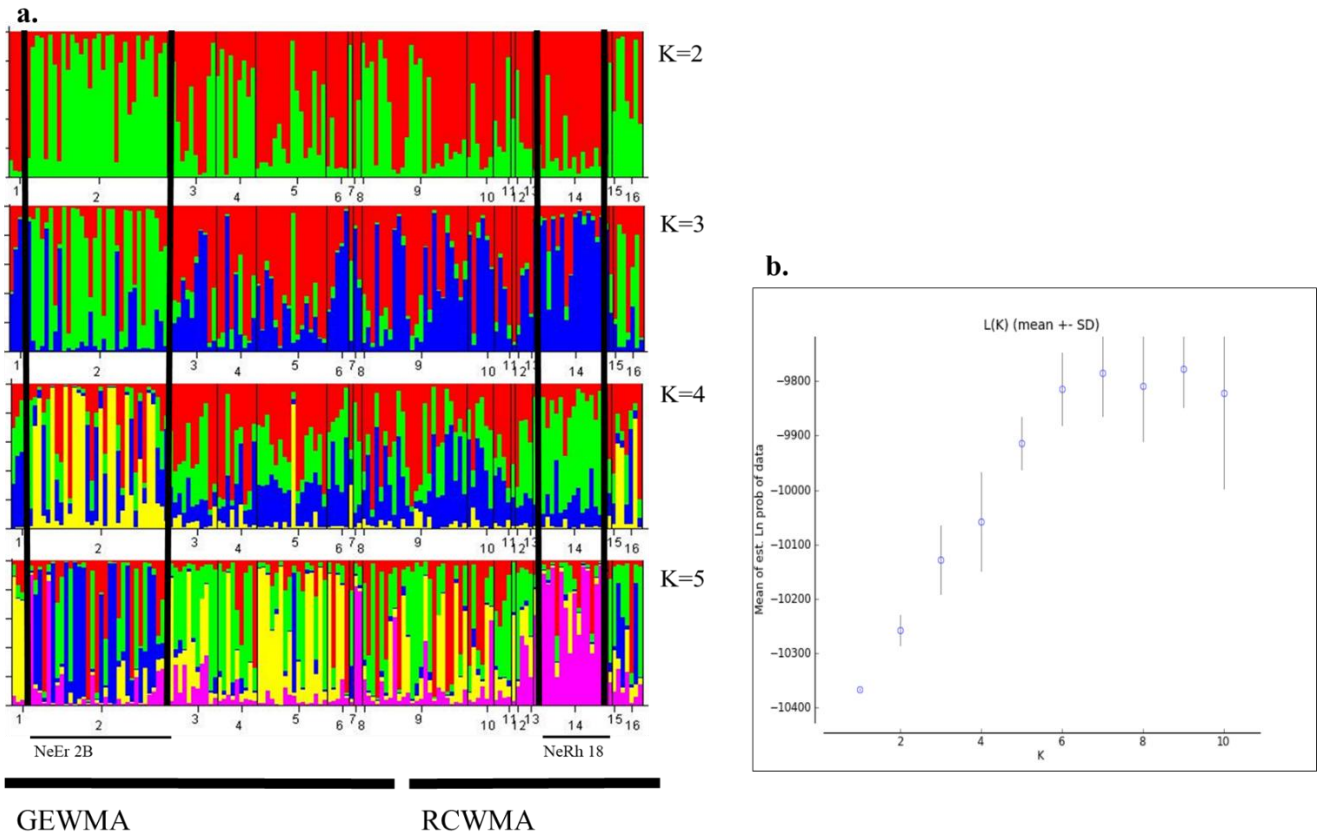


Figure 2.2. **a.** Results of the STRUCTURE analysis between *R. aniarum* individuals from Gus Engeling Wildlife Management Area and Richland Creek Wildlife Management area. No population subdivision is apparent between the two locations. Low but visible structure for infrapopulations is observable for host *Nerodia erythrogaster* 2B and for host *Nerodia rhombifer* 18 from K=2 to K=5. All other infrapopulations exhibit no visible infrapopulation structure **b.** Plot of the mean of the estimated natural log of the probability of the data,

Table 11.2. Global F_{ST}^{A} among infrapopulations of *R. aniarum* collected from *Nerodia spp.* hosts. Corrected F_{ST}^{N} values are based on estimates from the program FreNA which re-estimates F_{ST} values based on the estimated rates of null alleles within the data set. Significance values given above the diagonal using the Weir and Cockerham theta estimate (1984), 999 permutations.

	Among Infrapopulation	*P* value
F_{ST}^{A}	0.0256	0.001
F_{ST}^{N}	0.0265	0.001

Table 12.2. Pairwise measures of F_{ST} based on 9 microsatellite markers among hosts infected with more than a single worm (N=126). Host AgPi 20 was infected with a single *R. ancistrodontis* individual and was excluded from the analysis. Significance values given above the diagonal using the Weir and Cockerham theta estimate (1984), 999 permutations.

	AgPi 12	**AgPi 10**	**AgPi 11**	**AgPi 13**	**AgPi 14**	**AgPi 16**	**AgPi 17**	**AgPi 18**
AgPi 12		**0.001**	0.285	0.089	0.216	0.362	0.455	**0.001**
AgPi 10	0.142		0.027	**0.001**	0.005	**0.001**	**0.001**	**0.002**
AgPi 11	0.005	0.051		0.080	0.454	0.202	0.489	0.219
AgPi 13	0.011	0.115	0.007		0.104	0.195	0.271	**0.001**
AgPi 14	0.008	0.101	0.000	0.010		0.037	0.479	0.008
AgPi 16	0.002	0.086	0.003	0.003	0.011		0.138	0.008
AgPi 17	0.000	0.103	0.000	0.004	0.000	0.007		**0.001**
AgPi 18	0.070	0.124	0.006	0.038	0.035	0.020	0.059	

Table 13.2. Global F_{ST}^{A} among infrapopulations of *R. ancistrodontis* collected from *A. piscivorus* hosts. Corrected F_{ST}^{N} values are based on estimates from the program FreNA which re-estimates F_{ST} values based on the estimated rates of null alleles within the data set. Significance values given above the diagonal using the Weir and Cockerham theta estimate (1984), 999 permutations.

	Among Infrapopulation	*P* value
F_{ST}^{A}	0.0133	0.001
F_{ST}^{N}	0.021	0.001

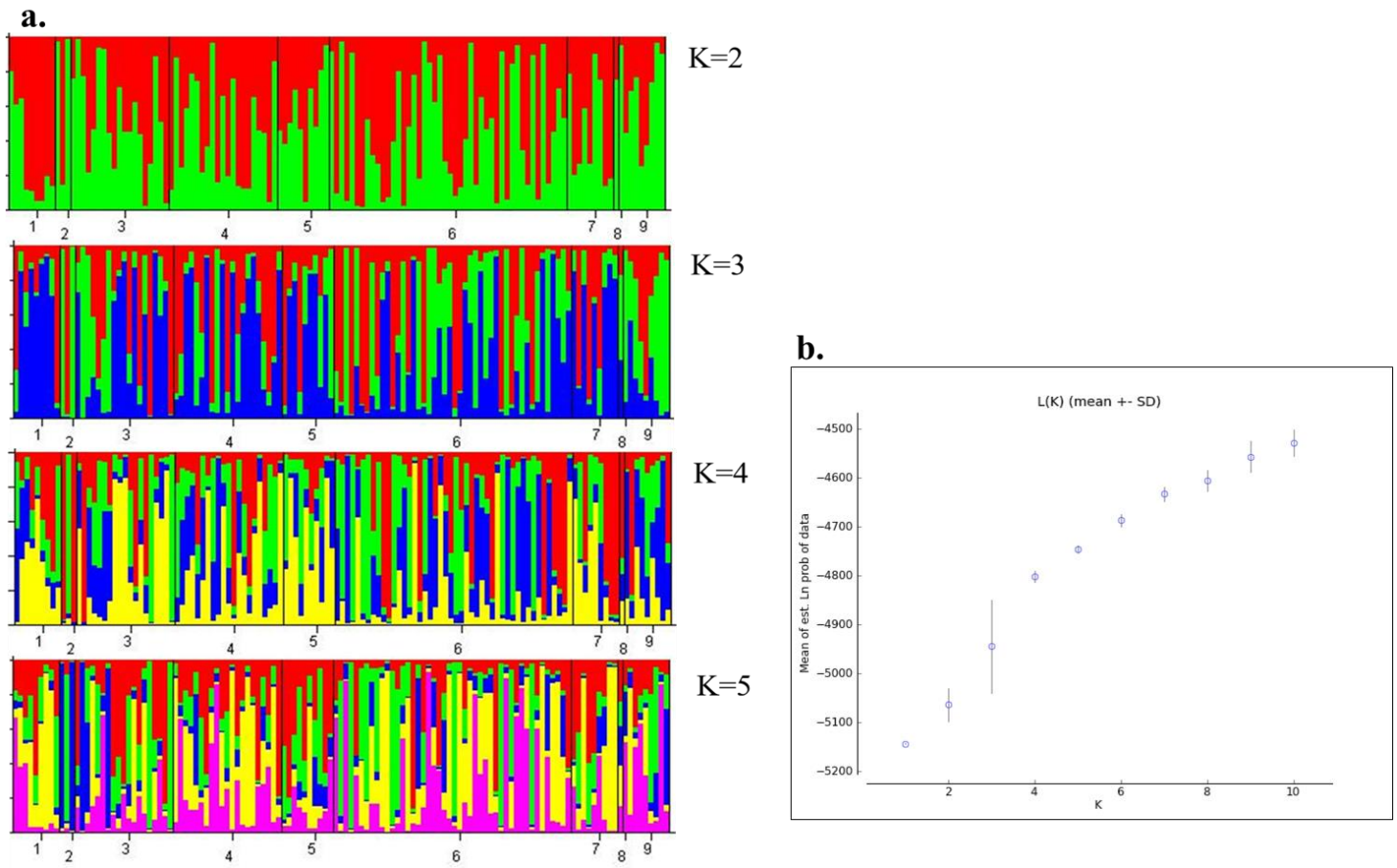


Figure 3.2 a . Results of the STRUCTURE analysis for *R. ancistrodontis* infrapopulation structure. Infrapopulations exhibit no visible infrapopulation structure at each K value**. b**. Plot of the Mean of the estimated natural log of the probability of the data.

Table 14.2. Effective population size estimates for *Renifer aniarum* using LDN*e* method for GEWMA and RCWMA and 12 microsatellite loci. Lowest allele frequency cutoff was 0.01 based on the guidelines of Waples and Do (2010).

Species	Location	N	r^2	N_e	Confidence Intervals
R. aniarum	GEWMA	87	0.01356	287.1	238.0- 359.1
	RCWMA	57	0.02282	307.4	221.8 - 489.4
R. ancistrodontis	RCWMA	127	0.00909	385.5	281.1-594.6

Table 15.2. Effective population size estimates from COLONY for *R. aniarum* and *R. ancistrodontis.* Parameters were set to male and female polygamy with inbreeding for a monoecious species, length of run was very long under the full likelihood method with very high precision, three runs, allele frequencies estimated from the data set and updated as the analysis was run Sibship scaling was set to yes and no sibship prior was assumed. Mutation/error rate was help constant. Run 1 (GEWMA[1] and RCWMA[1]) used an allelic dropout rate of 0.005, and Run two (GEWMA-INEst and RCWMA-INEst) used allelic dropout rates estimated in INEst 2.2.

Species	Location	N	*Alpha*	N_e	Confidence Intervals
R. aniarum	GEWMA[1]	87	0.21	115	81-157
	GEWMA-INEst	87	0.15	91	66-130
	RCWMA[1]	57	0.09	108	74-160
	RCWMA-INEst	57	0.10	81	57-119
R. ancistrodontis	RCWMA [1]	127	0.45	297	237-383
	RCWMA-INEst	127	0.46	247	197-316

Effective Population Size

The effective population size was calculated in three ways for each species: 1) LDN*e*. 2). COLONY parameter set one and 3) COLONY parameter set 2 with allele dropout rates estimated in INEst. Effective population size estimates for *R. aniarum* varied most between methods, ranging in size from 307 for RCWMA based on LDN*e* to 89 in the estimation from COLONY (Table 14.2 and Table 15.2). Effective population sizes for *R ancistrodontis* varied from 385 from LDN*e* to 247 in the estimation from Colony. Estimates of effective population size were similar between the two populations of *R aniarum* at GEWMA (105) and RCWMA (89). Based on the estimates of effective population size in COLONY with estimates of null allele frequency estimated in INEst, the effective population size of *R. ancistrodontis* in RCWMA was similar in size or slightly larger than the effective size of *R. aniarum* at the same location.

Phylogenetic Reconstructions and Mitochondrial Diversity

The CO1 gene (~940 bp) was sequenced for 33 *R. ancistrodontis* individuals and 36 *R. aniarum* individuals (23 from RCWMA and 13 from GEWMA). Three *R. aniarum* individuals from GEWMA yielded low quality DNA and were exclude from the analysis, for a total of 69 sequenced individuals. Branch length and monophyly between *R. aniarum* and *R. ancistrodontis* indicates that the two species are phylogenetically distinct but masks the different levels of diversity between the two groups (Figure 4.2). Trees were then constructed for each species separately. The phylogenetic analysis of *R. aniarum* suggests that there is qualitatively no geographically dependent separation

between individuals collected from the two locations as individuals from each location are randomly distributed throughout the tree (Figure 5.2). The results of an AMOVA between GEWMA and RCWMA using the CO1 data supported the lack of differentiation between GEWMA and RCWMA at the mitochondrial level ($Phi\text{-}_{ST}$=-0.0478, X^2=16.66, *d.f.*= 18 *p*=0.546). The phylogenetic analysis for *R. ancistrodontis* revealed two distinct clades (Figure 6.2). Branch length and 6% percent difference between the two clades are suggestive of two cryptic lineages, however no corresponding pattern was found in the microsatellite data.

Nucleotide diversity statistics calculated for the COI sequence are given in Table 16.2. When all of the sequenced individuals from both species are included in the analysis number of variable sites, Theta per sequence, theta per site, nucleotide diversity, and average number of nucleotide differences were all moderately higher in *R. ancistrodontis* than *R. aniarum*. However, when the two individuals from the red clade (Figure 6.2) are removed from the *R. ancistrodontis* data set, all measures of nucleotide diversity for *R. ancistrodontis* decrease (Table 16.2). High levels of nucleotide diversity in *R. ancistrodontis* is driven largely by the presence of these two divergent individuals.

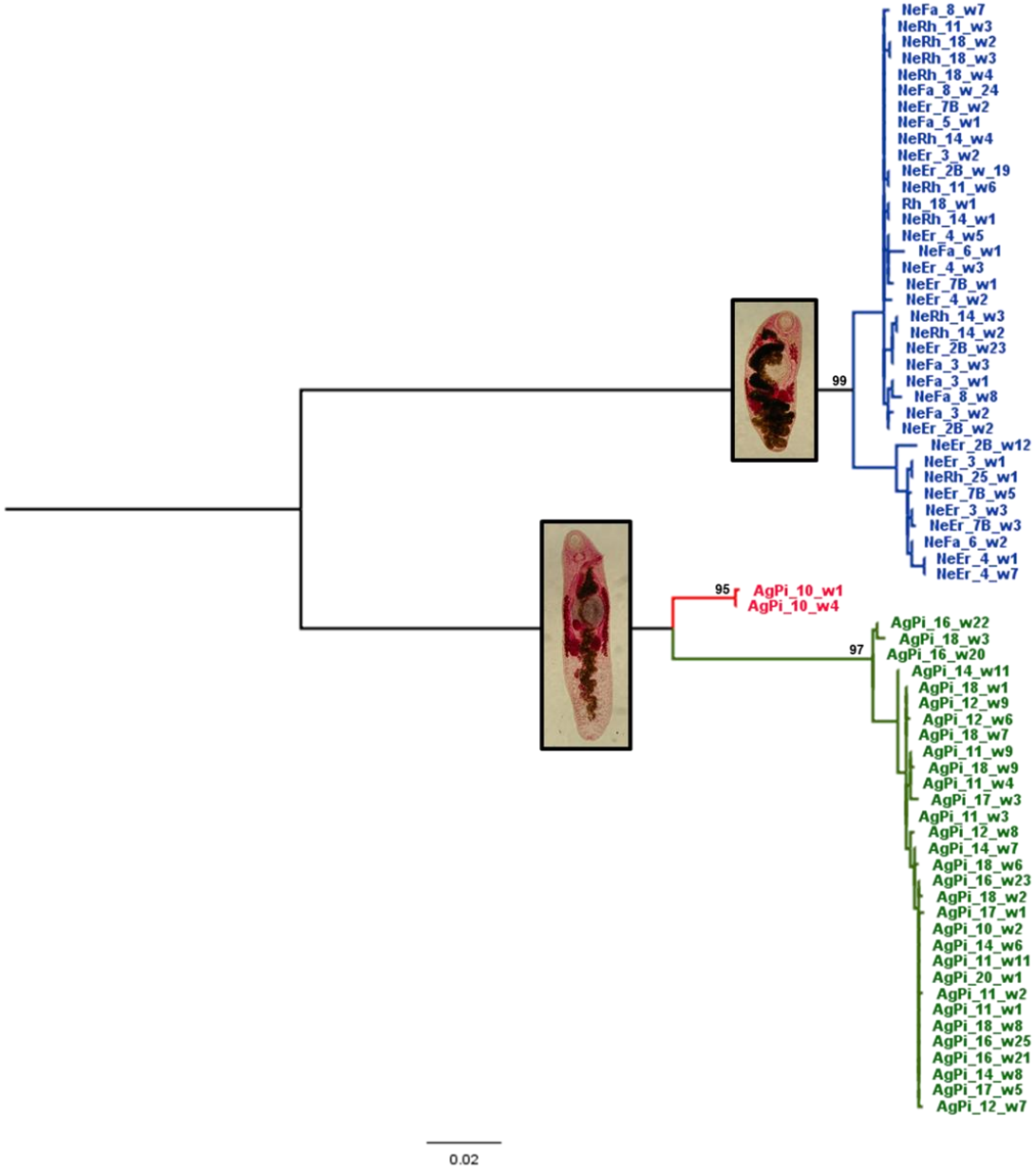


Figure 4.2 Phylogeny constructed from cytochrome oxidase subunit 1 (CO1) gene (~940 bp) of a subset of the genotyped individuals within the sample set, including 13 *R. aniarum* from GEWMA , 23 from RCWMA, 33 *R. ancistrodontis* from RCWMA. Maximum likelihood phylogeny was from model HKY+G was obtained in MEGA-X. Statistical support is provided above nodes. Host species name abbreviations (NeEr: *N. erythrogaster*, NeRh: *N. rhombifer*, *N. fasciata*, AgPi: *A. piscivorous*) indicate the host species from which the worm was removed.

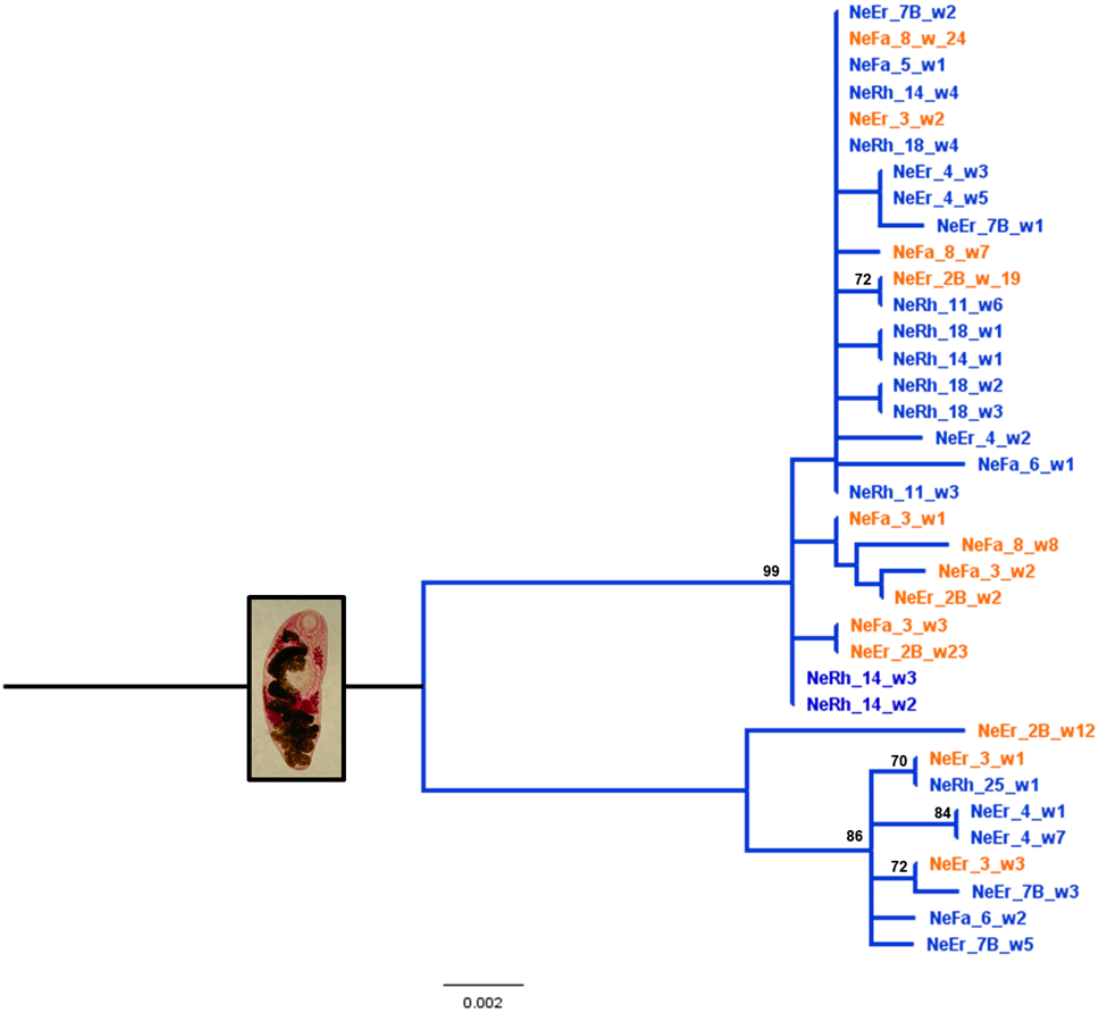


Figure 5.2. Phylogeny constructed from cytochrome oxidase subunit 1 (CO1) gene (~938 bp) of a subset of the genotyped individuals within the sample set, including 13 *R. aniarum* from GEWMA (Orange), 23 from RCWMA (Blue). Maximum likelihood phylogeny from model HKY was obtained in MEGA-X. Bootstrap values of 70 or greater are given at nodes. Host species name abbreviations (NeEr: *N. erythrogaster*, NeRh: *N. rhombifer*, *N. fasciata*) indicate the host species from which the worm was removed.

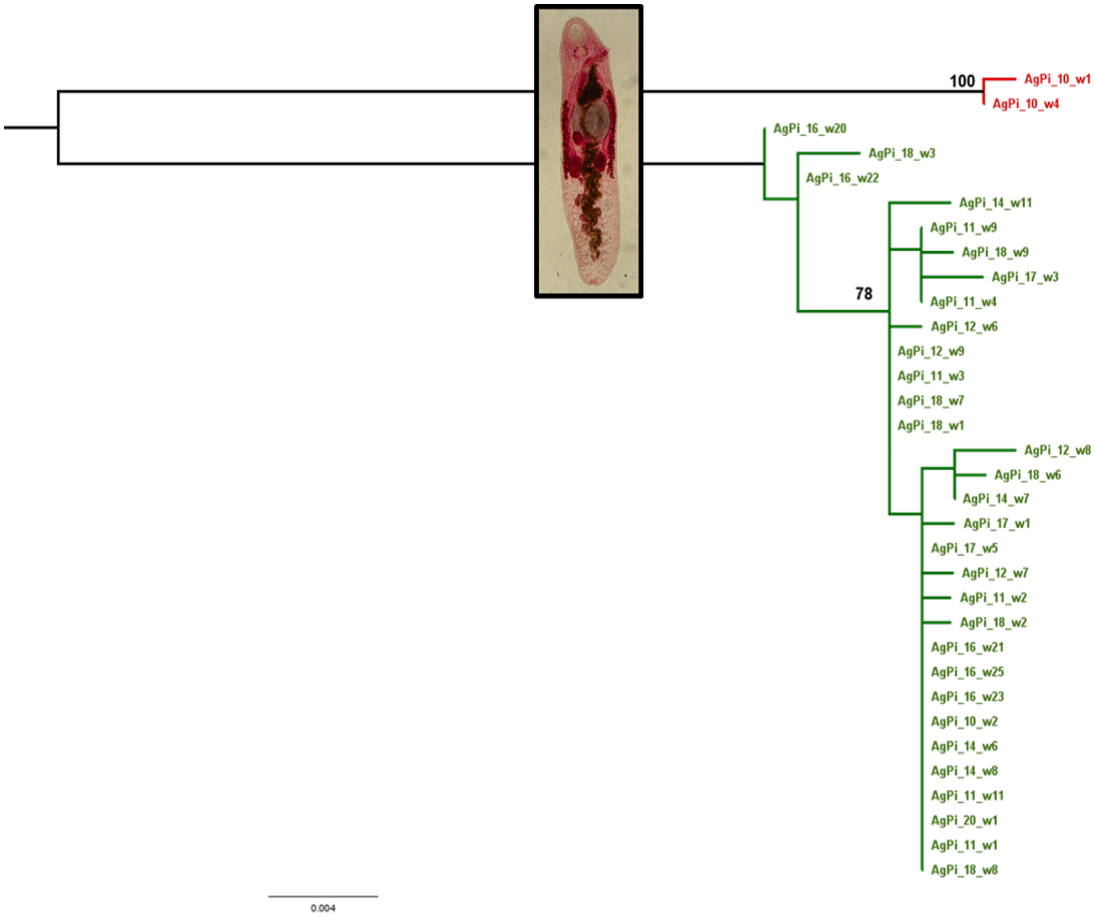


Figure 6.2. Phylogeny constructed from cytochrome oxidase subunit 1 (CO1) gene (~940 bp) of a subset of 33 *R. ancistrodontis* Maximum likelihood phylogeny from model HKY was obtained in MEGA-X. Statistical support is provided above nodes. Statistical support is provided above nodes. Host species name abbreviations (AgPi: *A. piscivorous*) indicate the host species from which the worm was removed.

Table 16.2. Mitochondrial diversity observed in ~940 bp of the COI gene for *R. aniarum* and *R. ancistrodontis* populations.

Species	N[a]	Vs[b]	N_{HAP}[c]	D_{HAP}[d]	SD[e]	π[f]	SD[e]	K[g]	Theta per sequence	Theta per site	Tajima's D
R. aniarum	36	38	18	0.900	0.042	0.00944	0.0015	8.14	9.40488	0.01090	-0.47817 (p=0.10)
R. ancistrodontis	33	67	17	0.860	0.054	0.01	0.0057	8.59	16.75	0.01967	-1.81 (p<0.05)
R. ancistrodontis	31	21	17	0.867	0.055	0.002	0.0004	2.43	5.25	0.00614	-1.89633 (p<0.05)

[a] Number of individuals sequenced
[b] Variable sites
[c] Number of haplotypes
[d] Haplotype diversity
[e] Standard Deviation
[f] Nucleotide diversity
[g] Average number of nucleotide differences

Discussion

Nadler (1995) predicted that parasites that use multiple species of definitive hosts would exhibit little to no population structure and greater genetic diversity than their host specific counterparts. Use of multiple host species was predicted to facilitate gene flow via increased definitive host dispersal, maintain a larger effective size, and reduces the probability of local extinction events. We tested the predictions of this hypothesis using a co-structure analysis *R. aniarum* (generalist) and *R. ancistrodontis* (host specific) at the local scale. Contrary to the predictions of Nadler's hypothesis, measures of microsatellite diversity of both species were high and not significantly different. *R. aniarum* exhibited low population subdivision between GEWMA and RCWMA at either the microsatellite or mitochondrial level. The high levels of diversity in *R. ancistrodontis* are driven primarily by two divergent individuals. At the infrapopulation level, both *R. aniarum* and *R. ancistrodontis* exhibited little among host genetic structure.

The microsatellite estimate of effective population size of the host specific *R. ancistrodontis* was slightly larger than the effective size of the generalist *R. aniarum.* Again these results do not support the predictions of Nadler's hypothesis. The reasoning behind this prediction is that the cumulative number of hosts available to a generalist parasite are capable of supporting larger parasite populations. It is possible then that *A. piscivorus* population sizes in the Richland Creek Area are larger than the cumulative population sizes of the *Nerodia* host species, and capable of supporting larger parasite populations. Ryberg et al. (2004) documented that both *N. rhombifer* and *A. piscivorus*

exhibited high relative abundance in Richland Creek WMA, while *N. fasciata* and *N. erythrogaster* relative abundance was low. The relative abundances of definitive hosts at GEWMA are unknown. Future investigations to quantify definitive host abundance could prove useful in delimited host-abundance driven differences in *R. aniarum* and *R. ancistrodontis* effective sizes.

Taken together, the results of this study did not find support for the predictions of Nadler's hypothesis at the local-scale for *R. aniarum* and *R. ancistrodontis*. This represents the first study to examine this hypothesis through the use of a co-structure analysis to find patterns of genetic diversity, substructure and relative effective size contrary to this hypothesis. However, in addition to differences in spatial scale, there are some important differences in the system in which the hypothesis were addressed that may have contributed to our results. Both Johnson et al (2002) and Martinu et al (2018) studied ectoparasites with direct transmission. Falk and Perkins (2013) focused on two species of nematode whose transmission is also presumed to be direct via fecal-oral contact. In contrast, *R. aniarum* and *R. ancistrodontis* exhibit complex, semi-aquatic life cycles and infect hosts with significant dispersal abilities. The additional complexity of using multiple host species at each life cycle stage (snails, tadpoles and snakes) combined with the potential for high levels of mixing in the aquatic environment may work to uncouple the patterns of genetic structure predicted by differences in host specificity. The first intermediate snail host may influence *R. ancistrodontis* and *R. aniarum* dispersal during seasonal flooding events within the Trinity River flood plain. Ryberg et al. (2004) in a vertebrate inventory of RCWMA found high relative

abundances of the second intermediate amphibian hosts, which may also facilitate *R. ancistrodontis* dispersal, especially if infective metacercaria as retained in adult frogs. *Agkistrodon piscivorus* has been documented to move between seasonally flooded wetlands as their availability shifts in response to drought in addition to seasonal migrations between wetland and upland habitats (Willson 2006; Glaudas et al. 2007; Kirkly 2018). Mathee et al. (2018) examined the predictions of SGVH (i.e. Nadler's hypothesis developed for free-living organisms) in two species of host specific mites. They found that the host specific mite species with greater dispersal showed less population subdivision and greater genetic diversity than the host specific louse. The author's suggest that the predictions of Nadler's hypothesis be modified to apply only to host specific parasites with limited dispersal abilities. The results of our study concur with the assertion of Matthee et al. (2018) that Nadler's hypothesis will not be applicable to host-specific parasites with greater dispersal abilities.

Interestingly, the primary difference in the genetic characteristics of the two species was the higher estimates of F_{IS}, observed in *R. ancistrodontis*. While there was variation in the degree of selfing or inbreeding estimated by the different programs, higher F_{IS} values, or higher selfing rates were consistently associated with *R. ancistrodontis* compared to *R. aniarum*. This is especially unusual given the high levels of diversity documented in *R. ancistrodontis*, as pervasive inbreeding and selfing has been recorded to be associated with reduced genetic diversity in many taxa, including other parasite taxa (Charlesworth 2003; Detwiler and Criscione 2011; Detwiler et al. 2017). Little is known about the rates of inbreeding and selfing in monoecious wildlife

parasite populations or how these rates vary over time and space. Detwiler et al. (2017) determined that the distribution of infection intensities among hosts can alter the level of inbreeding within parasite populations, where greater intensities of infection result in lower selfing rates for *Oocharsitica javaensis* tapeworms infecting Mediterranean Geckos (*Hemidactylus turcicus*). *R. ancistrodontis* however had a mean intensity of infection of 15.33±4.37, which is greater than or equal to intensity of infection recorded among *R. aniarum* hosts, suggesting that low intensity of infection is not driving the high levels of inbreeding.

Conclusions

The results of this study did not support the predications of Nadler's hypothesis regarding the genetic consequences of host specificity. Rather they support the assertion of Mathee et al. (2018) that high levels of host dispersal may negate the genetic consequences of host specificity. Furthermore, complex life-cycles and aquatic mixing may further add to the high levels of genetic diversity and low population subdivision in the specialist *R. ancistrodontus*. As this is the first study to examine the predictions of Nadler's hypothesis for a trematode parasite with a complex life cycle and semi-aquatic transmission, additional research is required to determine if other host specific trematodes exhibit high genetic diversity and low population subdivision.

CHAPTER III

UNIDIRECTIONAL STREAM DRIFT, DENDRITIC ECOLOGICAL NETWORKS, AND HOST DISPERSAL: PARASITE GENE FLOW IN RIVERINE HABITATS

Introduction

River systems are characterized by their dendritic structure and unidirectional stream flow. The structural characteristics of river systems are known to influence the ecology and evolution of their inhabitants at multiple scales, from determining community composition to altering the genetic structure of their inhabitants (Barger and Esch 2001; Paz-Vinas and Blancet 2015). Rivers can serve as barriers to gene flow by isolating populations between drainage basins and impact the gene flow of aquatic invertebrate organisms through the process of unidirectional downstream drift (Allan and Castillo 2007;Thornton 2008). Stream drift is the downstream transport of organic matter, invertebrates and fish, caused or facilitated by the unidirectional current of lotic systems. Unidirectional stream drift can facilitate downstream dispersal and asymmetric migration rates for fluvial organisms, potentially generating a pattern of increased diversity in downstream reaches of the river if gene flow is similarly unidirectional (Paz-Vinas et al. 2013).

Dendritic ecological networks (DEN) are habitats characterized by repeated, arborescent bifurcations that form landscape pathways of branches and nodes (Campbell Grant et al. 2007). The branches of the network are set within a spatial hierarchy of headwaters, tributaries, reaches, and streams which may also serve as tributaries to larger

rivers further downstream. Connectivity along branches can be restricted both by the geographic placement of the population within the stream network but also by additional physical barriers such as waterfalls (Crispo et al. 2006; Vaha et al. 2007) or reservoirs (Atkinson and Bartholomew 2010: Castello and Macedo 2016). The bifurcating networks of riverine habitats can generate patterns of isolation by distance among stream networks, and population subdivision among river branches if dispersal among hierarchical branches is limited (Alp et al. 2012; Cuddington and Yodzis 2002; Labonne et al. 2008; Meffe and Vrijenhoek 1998; Paz-Vinas and Blanchet 2015; Paz-Vinas et al. 2015).

The importance of stream drift and DEN's in shaping the genetic population structure of invertebrate and vertebrate organisms depends largely on the dispersal ability of the organism in question. Stream drift has been documented to be a major force behind the dispersal of invertebrate organisms including snails, as well as small fish and tadpoles that serve as intermediate hosts for many trematodes (Blondel et al. 2018; Crispo et al. 2006; Poff et al. 1997). Populations may often exhibit patterns of endemism constrained within branches, isolation by distance in river networks and downstream-biased migration (Campbell Grant et al. 2007; Thomaz et al. 2016; Whelan et al. 2019). However, phoretic dispersal via birds and aerial adult aquatic insects has also been demonstrated to facilitate upstream and between stream dispersal (Chaput-Bardy et al. 2009; Tonkin et al. 2014). For parasites with no or short-lived free-living stages with limited dispersal, it is predicted that gene flow is largely dependent on host mobility (Nadler 1990; Prugnolle et al. 2005). For parasites with complex life cycles,

the most mobile host in the life cycle is predicted to exert the most influence on population connectivity (Blasco-Costa et al. 2012; Louhi et al. 2010; Prugnolle et al. 2005). Thus, hosts with high dispersal abilities may negate the influence of stream drift and DEN on trematode parasites by facilitating upstream dispersal. Previous research demonstrated the dispersal of parasites with fully aquatic definitive hosts (autogenic parasites) are restricted between river systems, while parasites with aquatic-to-terrestrial life cycles (allogenic parasites) have high gene flow between stream drainages (Blasco-Costa and Poulin 2013; Criscione and Blouin 2006;). Differences in host dispersal can be compounded by features of the landscape that have the potential to further restrict host movement and produce population subdivision through reduced gene flow (Allan and Castillo 2007). However, it remains unknown how semi-aquatic hosts that are tightly bound to aquatic systems such as water snakes may impact parasite gene flow along and among riparian systems.

To date a single study has examined the importance of stream drift and host dispersal in shaping parasite population structure (Blasco-Costa et al. 2012). Blasco-Costa et al. (2012) examined the genetic diversity of two parasites along a section of river in New Zealand using the mitochondrial cytochrome c oxidase subunit 1 (COXI) and 16S ribosomal genes. They determined the parasite *Coitocaecum parvum* exhibited isolation by distance and moderate genetic substructure as predicted by the stream drift hypothesis. However, in *Stegodexamene*. *anguillsa,* a parasite that infects New Zealand longfin eels (*Anguilla dieffenbachia*), showed no pattern of isolation by distance or

increase in genetic diversity with distance downstream. Thus infecting more mobile hosts can override the stream drift effect.

Based on their results, Blasco-Costa et al. (2012) concluded that parasites with less mobile hosts are more likely to be influenced by stream drift effect. However, further work is required to determine if this pattern can be generalized. Furthermore, because their investigation was conducted over a relatively short section of unbranched and uninterrupted river (~70 km), it is not known how the additional complexity of river confluences, longer distances and the contributions of tributaries may alter the observed results. In particular, genetic diversity is expected to increase below river confluences because isolated reaches of the dendritic system are joined (Paz-Vinas and Blanchet 2015).

The effects of DENs on parasites are similarly under examined. Pettersen et al. (2015) studied the population structure of the monogenean parasite *Gyrodactylus thymallii* along a large glacially influenced river in Norway using both cytochrome oxidase I (COI) and dehydrogenase subunit 5 (NADH 5). *Gyrodactylus thymallii* exhibits direct transmission with a simple one host life cycle, infecting Grayling throughout the Glomma River system. The authors determined that *G. thymalli* exhibited significant isolation by distance and population subdivision associated with the three main branches of the river driven by the presence of waterfalls, dams and differences in historical glaciations events. However, additional work is needed with fine-scale sampling and microsatellite markers to examine contemporary patterns of population subdivision within DEN for parasites with more mobile aquatic hosts.

The purpose of this investigation was to examine the influences of stream drift, dendritic ecological networks and host dispersal on the population structure of the parasite *R. aniarum* along the Upper Colorado and Concho River and their tributaries. *Renifer aniarum* infects all three species of semi-aquatic water snakes (*Nerodia erythrogaster, N. rhombifer* and *N. paucimaculata*) that inhabit the Colorado and Concho River and their tributaries. The Concho and Colorado Rivers are major waterways located in the semi-arid regions west of the Edwards Plateau in west-central Texas. Water snakes are known to disperse long distances and *N. rhombifer* and *N. erythrogaster* are known to disperse terrestrially (Gibbons and Dorcas 2004). Rodriguez et al. (2012) estimated population structure of *N. erythrogaster transversa* between the Concho and Colorado Rivers using cyt-*b* mtDNA and 5 microsatellite markers. They found limited population structure and high levels of genetic diversity among the three reaches of the Colorado and Concho Rivers, indicating there may be dispersal of the hosts between the river systems. Both of these host dispersal traits may eliminate or reduce the influence of stream drift and dendritic ecological networks on parasite population structure and facilitate parasite dispersal both up and down stream and between reaches of the river. Interpretations of the potential influences of these three factors are as follows:

If the physical characteristics of the river are the primary influence on *R. aniarum* population structure than I predict that one or both of these non-mutually exclusive results would be observed:

1. If *R. aniarum* population structure supports the predictions of the unidirectional stream drift hypothesis, then populations infecting the semi-aquatic snake hosts will increase in genetic diversity with an increasing distance downstream and exhibit moderate to high genetic structure among subpopulations. Additionally, populations downstream of the confluence of the Concho and Colorado Rivers should be more genetically diverse than upstream populations due to the contributions of the two separate upstream drainages.
2. If the physical structure of the DEN is a strong influence on *R. aniarum* dispersal, population subdivision will be associated with the structural features of the river system, specifically among the three reaches of the Colorado and Concho Rivers and the Elm Creek and Lipan Creek tributaries.

Alternatively, host dispersal may be the primary factor influencing *R. aniarum* population structure:

3. If host dispersal is the primary influence on *R. aniarum* population structure, than *R. aniarum* will exhibit no isolation by distance, a non-significant correlation between genetic diversity metrics and geographic distance and little to no population subdivision among reaches of the river, as the most mobile definitive host exhibits no population subdivision along these reaches.

Methods

Survey sites and Sample Collection

Parasites were collected from 11 locations along the Colorado and Concho Rivers, one location on Lipan Creek (a tributary to the Concho River) and one location

on Elm Creek (a tributary to the Colorado River) for a total of 13 collection locations. The Concho and Colorado Rivers form a confluence within O.H. Ivie Reservoir, separating the river system into three reaches, the Upper Colorado River above O.H. Ivie reservoir, the Lower Colorado River downstream of O.H. Ivie reservoir, and the Concho River (Figure 1.3). Sites were selected to encompass locations on the three reaches of the river and two tributaries. Site location was based in part on accessibility, network placement and in part to coincide with post-delisting monitoring surveys of the Concho water snake, *N. h. paucimaculata*. At each collection site, the snake hosts were captured by hand or with the use of partially submerged minnow traps baited with Little Stinker catfish bait. Parasites were removed from the mouth, heat-killed under a cover slip and placed in 70% EtOH for use in the molecular analysis. Snakes were processed within 24 hours of capture and returned to their original location.

Microsatellite Genotyping and Analysis

DNA was extracted from a subset of the *R. aniarum* individuals collected (Table 1.3). Individuals were removed from multiple hosts to increase the likelihood obtaining samples representative of the overall population diversity (as opposed to infrapopulation diversity). DNA was extracted by placing the anterior portion of the worm in 200 uL of 5% chelex and 0.2 mg/ml of protinase K, incubated for 2 hours at 56ºC and boiled at 100ºC for 10 min. PCR amplification was performed for markers described in Chapter II in 10 µL reactions containing 3.1 µL ultrapure water, 5 µl 2X Qiagen Type-IT kit Master Mix, 0.16 µL fluorescent-labeled M13 primer (Applied Biosystems: FAM), 0.08 µL M13-labeled forward primer, 0.16 µL of 10 µM reverse primer and 1.5 µL of

genomic DNA. The thermocycler profile was 94 ºC for 5 minutes, 31 cycles of 94 ºC for 30 seconds, 56 ºC for 45 seconds, 65 ºC for 45 seconds, followed by nine cycles of 94 ºC for 30 seconds, 53 ºC for 45 seconds 65 ºC for 45 seconds and extension at 65 ºC for 10 minutes. PCR-product was visualized on a 2% agarose gel run in 0.5X TBE buffer at 95 ºC for 45 minutes. Reactions that yielded discrete bands in the expected product size range were sent to the DNA Analysis Facility on Science Hill at Yale University (New Haven, CT, USA) and visualized on a 3730xl 96-capilary Genetic Analyzer with 500-LIZ size standard. Samples were genotyped on GeneMarker 2.6.4. Marker ReAn 5219 was not amplified in the Colorado and Concho River samples due to high failure rate in these individuals during the initial screening process.

Identification of Clonemates

Multilocus genotypes (MLGs) were assessed using GENALEX 6.502 in order to identify potential clonemates (genetically identical individuals) that may be present due to asexual reproduction in the snail host. Given the high diversity of the loci it is extremely unlikely that unique clones (i.e., individuals that are the product of sexual reproduction) would have identical multilocus genotypes. One representative of each clone was included in downstream analyses to assess the prior adult generation's mating system and to prevent artificial inflation of population structure due to clonemates (Criscione et al. 2011: Prugnolle et al. 2005).

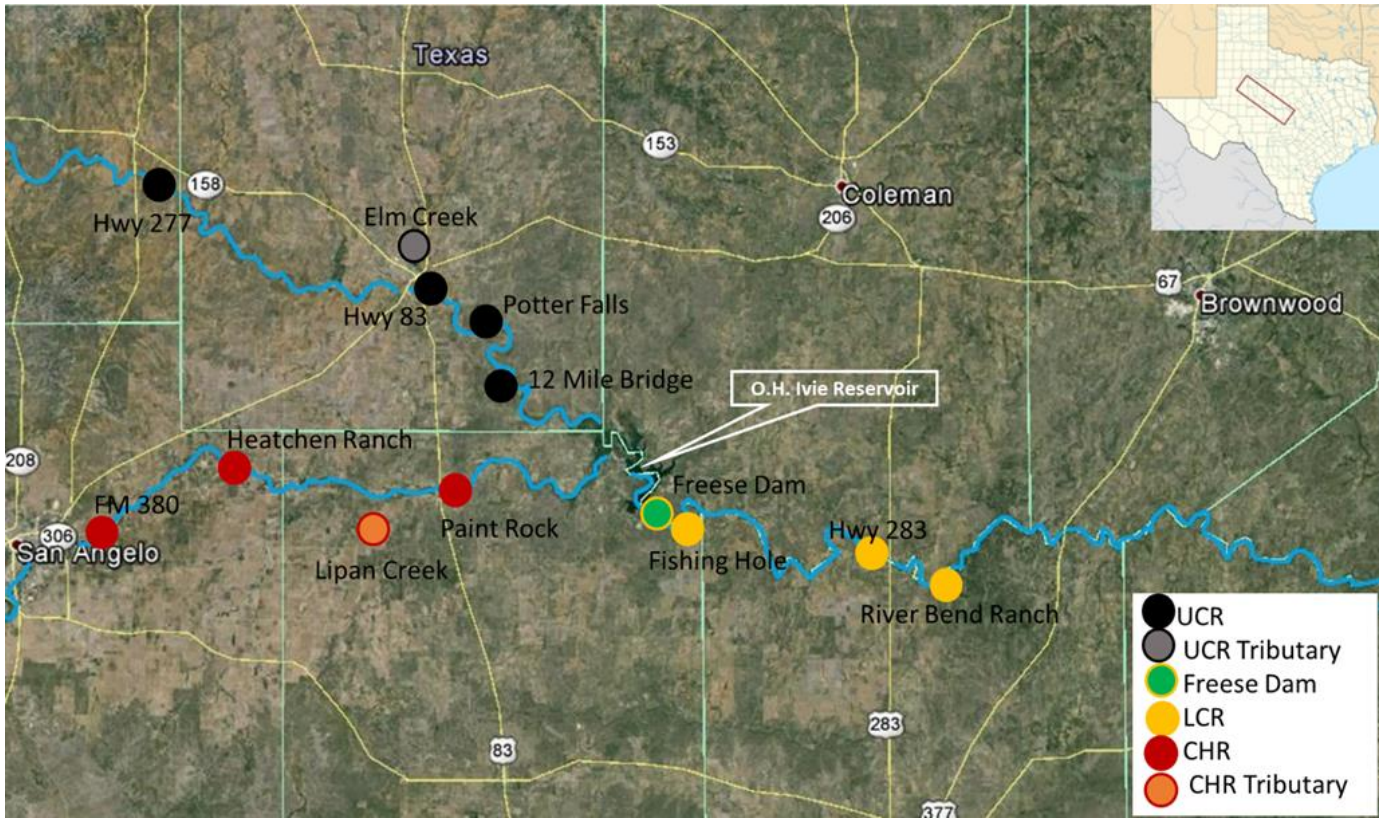


Figure 1.3. Map of collection sites for *R. aniarum* along the Upper Colorado River (UCR), Lower Colorado River (LCR) and Concho River (CHR) in west-central Texas, USA. (Google Earth Pro Images Landsat/Copernicus 2018).

Genetic Diversity and Disequilibrium Tests

Genotypic disequilibrium for pairs of microsatellite loci was tested using GENEPOP web 4.2 (Raymond and Rousset 1995). Statistical significance was determine using the log likelihood ratio statistic with Markov chain parameters all set for 5000 dememorizations, batches and iterations per batch. Gene diversity (H_S), the number of alleles per locus (*An*), and allelic richness (*AR*) (rarefied number to smallest sample size of N =19) were calculated in R (R Core Development Team 2017) package ade4 and heirfstat (Goudet 2005). Estimates of F_{IS} (which quantifies the proportional change in heterozygosity due to deviations in Hardy Weinberg equilibrium, HWE) were calculated in FSTAT 2.39 (Goudet 2001).

Straight-line Distance, River Distance and Genetic Diversity

The stream-drift hypothesis predicts a negative correlation between genetic diversity measures and distance relative to the most downstream collection site. We determined if *R. aniarum* exhibited an increase in genetic diversity with an increase in distance downstream by examining the correlation between river distance relative to the most downstream network position against allelic richness (*AR*), and gene diversity (H_S). Gene diversity measures did not meet assumptions of normality and were arcsine square root transformed prior to the correlation analysis (Whelan et al. 2019). Definitive host species (*Nerodia spp*.) are potentially capable of both terrestrial and aquatic dispersal and so have the potential to transmit *R. aniarum* infections through out-of-network host movement (i.e. terrestrial travel). To determine which of these potential dispersal paths was most associated with the distribution of *R. aniarum* genetic diversity, correlations of

straight-line distance (shortest linear distance between collection sites which can include out-of-network travel between collection sites as calculated by Euclidean distances) and river distance (distance between sites following network pathways) for each of the response variables. River distances between collection sites were measured by tracing river network path between sites in Google Earth Pro (Google 2018). Distances were measured from the most downstream location of one site to the most upstream point of the next site. Network position was measured as distance from the most downstream site on the Colorado River (River Bend Ranch) as described by Paz-Vinas et al. (2015). Straight-line distance between the centers of each collection site were calculated in adegenet 2.1.1 (Jombart 2008). Correlations were carried out in Excel. Statistical tests for differences in average genetic diversity indices between locations and between reaches of the river were examined using the non-parametric Kruskal-Wallis test and implemented in R using the heirfstat, dbplyr and coin libraries.

Population Structure and Geographic Distance

Population structure between parasites collected at different location sites was assessed by calculating pairwise F_{ST} values and statistical significance values between each collection location was carried out in heirfstat (Goudet 2005). Principal Coordinate Analysis (PCoA) was performed in GENALEX 6.502 (Peakall and Smouse 2012) was used to visualize differences in pairwise F_{ST} values among collection sites. Mantel tests for correlation between river distance and pairwise linearized F_{ST} values and straight-line distance and pairwise F_{ST} values were calculated in GENALEX with 999 random permutations (Peakall and Smouse 2012). Euclidean straight-line distanced for Mantel

tests were log transformed following the recommendations of Rousset (1997) for two dimensional habitats. I used the Bayesian clustering model based clustering implemented in STRUCTURE 2.3.4 (Pritchard et al. 2000). The input parameters for STRUCTURE were uncorrelated allele frequencies, and the no admixture model. STRUCTURE was run with 5,000,000 iterations with a burn-in of 500,000 iterations for K (i.e., the number of possible clusters) values 1 to 13 with 10 replications of each possible K value. Analysis of the PCoA plot, F_{ST} estimates and STRUCTURE analysis indicated that patterns of genetic structure on the main stem of the Colorado River and Concho River were masked by outlier populations in the tributaries and at Freese Dam. Mantel tests for isolation by distance were further examined in three reduced data sets 1). Only main-stem river collection sites with Freese Dam populations excluded from the data set, 2). Main-stem Concho and lower Colorado Sites excluding Freese Dam and 2). Main-stem upper Colorado and Lower Colorado sites excluding Freese Dam.

Table 1.3. Collection site locations and number of *R. aniarum* individuals included in the analysis

Collection Site	Number of snakes captured	Number of Parasites collected	Prevalence	Intensity ± SE	Number of parasites genotyped	Number of Individuals with unique MLGs [A]	Latitude	Longitude	Distance from RBR (km)[B]
Upper Colorado River									
Hwy 277	20	59	0.65	4.53±3.59	39	38	31.836247	-100.276314	172.87
Elm Creek [C]	10	116	0.9	12.88±3.59	42	38	31.785278	-99.946308	125.08
Hwy 83	25	109	0.56	7.78±1.54	35	34	31.729783	-99.926975	113.86
Potter Falls	13	56	0.84	5.09±1.31	50	43	31.67923	-99.84144	100.73
12 Mile Bridge	31	107	0.32	10.7±3.79	31	31	31.646584	-99.856775	95.2
Lower Colorado River									
Freese Dam	58	274	0.603	7.8±1.82	98	90	31.497855	-99.658048	57.8
Fishing Hole	10	134	0.9	14.88±5.12	33	30	31.47783	-99.63657	55.9
Hwy 283	41	134	0.43	7.44±2.17	46	44	31.44362	-99.38671	9.1
River Bend Ranch	18	164	0.77	11.71±2.09	51	47	31.41027	-99.31281	0
Concho River									
FM 380	9	75	0.888	9.375±2.99	23	19	31.47478	-100.33334	154.76
Heatchen Ranch [D]	9	92	0.667	15.33±6.14	23	23	31.47262	-100.14069	121.36
Lipan Creek	7	24	0.714	4.8±1.68	23	22	31.47368	-100.33517	127.46
Paint Rock	17	108	0.647	9.81±2.71	89	70	31.51047	-99.90667	99.36
Total	**268**	**1452**			**583**	**529**			

Results

Parasite Collections, Genetic Diversity and Disequilibrium Tests

A total of 268 water snakes were captured and 1,452 *R. aniarum* individuals were collected during the 2013-2015 sampling seasons from the 13 collection sites (Table 1.3). Eleven microsatellite markers were amplified for 583 *R. aniarum* individuals collected at 13 sites along the Colorado and Concho Rivers and the two tributary locations. Clonemates from the 583 individuals identified by identical multilocus genotypes were reduced to a single representative of each clone for subsequent analyses. Clonemates were identified primarily within the same host individual but in a few instances were identified in two different hosts caught in the same collection location. The remaining data set included in the analysis included 529 *R. aniarum* individuals from the 13 collection locations (Table 1.3). The number of loci in each population that exhibited significant deviations from HWE ranged from 2 to 6 (6 from Potter Falls). The alleles that exhibited significant deviations varied between collection sites, no markers exhibited significant deviations from HWE at all locations. For 11 microsatellite markers there are 55 pairwise comparisons between loci. At a nominal level of 0.05, one would expect 2.75 loci to be in LD by chance alone. Number of pairwise comparisons between loci that exhibited linkage disequilibrium differed between populations, ranging from 1 (Elm Creek) to 13 (Freese Dam). Freese Dam, Lipan Creek and Paint Rock collection sites had the greatest number of loci in linkage disequilibrium. Overall levels of gene diversity were high in the Colorado, Concho and tributaries (Table 2.3) and was not significantly different among the 13 locations based

on the Kruskal Wallis test ($X^2_{(12)}$ =1.703, p=0.999) or between the three reaches of the river ($X^2_{(2)}$ =0.751, p=0.6867). Observed heterozygosity was also high and not significantly different among the 13 collection sites ($X^2_{(12)}$=8.5939, p=0.7372) or between the three reaches of the river ($X^2_{(2)}$ =1.7203, p=0.4231). Allelic richness was similarly high and not significantly different among groups ($X^2_{(12)}$=1.3983, p=0.999) or among the three reaches of the river ($X^2_{(2)}$ =0.169, p=0.918). Multi-locus F_{IS} values ranged from 0.051 to 0.164 between collection location (Table 2.3). The number of private alleles ranged from one to seven. Sites with the largest number of private alleles were Hwy 83 (5) and 12 Mile Bridge (7) on the Upper Colorado River and Paint Rock on the Concho River. The tributary sites, Lipan Creek (0 private alleles) and Elm Creek (1 private allele), did not have more private alleles than sites located along the main-stem of the river.

Straight-line Distance, River Distance and Genetic Diversity

Correlations between expected heterozygosity and river distance and expected heterozygosity and straight-line distance were not significantly different for the full data set or the dataset excluding tributary and Freese Dam collection sites (Table 3.3 and Table 4.3). Near identical levels of genetic diversity measures among sites likely precluded our ability to determine if straight line distance or river distance was more closely associated with measures of genetic diversity. Correlations between allelic richness and river distance were significant. The overall patterns of significance remained the same when the tributary locations and the Freese Dam sites were excluded from the analysis, but correlations between allelic richness and river distance were

stronger (R^2=0.87, p=0.00008) for the reduced data set (Table 4.3). River distances had consistently higher R^2 values compared to straight-line distances (Tables 3.3 and 4.3).

Population Structure and Geographic Distance

Pairwise comparisons of F_{ST} values between sites were low for all sites, regardless of placement within the river network of between three reaches of the river but several comparisons tested significant (Table 5.5). The sampling site on FM 380, was the most upstream location on the Concho River and had significant differentiation from all other sites. Potter Falls also had significant levels of population subdivision between all populations except the nearest upstream site, Hwy 83 and the nearest downstream site, 12 Mile Bridge. The site at Freese Dam, located directly below the Outlet from O.H. Ivie reservoir exhibited significant population substructure between all other locations. The principal coordinate analysis visualization emphasizes the differentiation between Freese Dam and all other collection sites. The greatest principal components axis comparison explains 57% (1vs 2) of the variation in pairwise F_{ST} values among collection sites. Sites along the mainstream Upper Colorado River form a cluster. Sites from the main stem of the Concho River are dispersed along x axis in the lower right coordinate in accordance with their network position, with the most upstream site falling farthest to the right of the axis and the most downstream site located nearest the central axis. The lower Colorado River main-stem sites are located centrally and clustered with the Elm Creek and Lipan Creek Tributary sites. The results of the STRUCTURE analysis for K=3 through K=5 are given in Figure 3.3. Concordant with the results of the pairwise F_{ST} comparisons and the PCoA, the Freese Dam collection site

is dominated by a single genetic cluster at each K value. Unlike the PCoA and the pairwise F_{ST} values, the STRUCTURE analysis made no *a priori* assumptions about population assignment but still identified Freese Dam as a distinct population cluster despite being only 2 km upstream from the next nearest collection site (Fishing Hole). Some additional population substructure is visible between tributary and mainstream collection sites. No population substructure is visible among the three reaches of the river. The results of the mantel test for patterns of isolation by distance among all populations was not significant, for either river distance (p=0.161) or straight line distance (p=0.353) (Figures 4.3 and 5.3). However when the tributary locations and Freese Dam were excluded from the analysis, the results of the Mantel Test indicated significant isolation by river distance (R^2=0.156, p=0.045) (Figure 6.3) but not straight-line distance (Figure 7.3). Main stem sites (excluding Freese Dam) located along a linear pathway from the upper Colorado River and lower Colorado River did not exhibit isolation by distance (R^2=0.0025, p=0.479) (Figure 8.3), nor did sites located along a linear pathway from the Concho River to the Lower Colorado River (again excluding Freese Dam) (R^2=0.01574, p=0.140) (Figure 9.3). Main stem sites located along bifurcating network pathway on the upper Colorado River and the Concho River did exhibit significant isolation by distance (R^2= 0.47, p= 0.007) (Figure 10.3). Thus, significant isolation by distance patterns among sites are driven by isolation by distance patterns between sites located on the separate branches of the upper Colorado and Concho Rivers.

Table 2.3. Microsatellite diversity of *R. aniarum* populations at 11 loci from 11 collection sites on the Colorado and Concho Rivers and 2 tributary locations.

Population	PA^a	A_R[b]	H_O[c]	H_S[d]	F_{IS}[f]
Upper Colorado River					
Hwy 277	1	9.52	0.711	0.79	0.123
Elm Creek	1	10.19	0.703	0.803	0.113
Hwy 83	5	9.98	0.728	0.805	0.111
Potter Falls	1	9.97	0.687	0.807	0.161
12 Mile Bridge	7	10.133	0.6709	0.8031	0.181
Lower Colorado River					
Freese Dam	3	9.65	0.6741	0.786	0.149
Fishing Hole	3	10.06	0.7114	0.777	0.102
Hwy 283	3	10.37	0.7607	0.799	0.060
River Bend Ranch	3	10.90	0.613	0.8131	0.087
Concho River					
FM 380	1	9.60	0.7033	0.792	0.140
Heatchen Ranch	1	9.84	0.7166	0.7968	0.123
Lipan Creek	0	10.06	0.677	0.818	0.098
Paint Rock	4	9.93	0.733	0.805	0.097

[a] Number of private alleles

[b] Allelic richness

[c] Observed heterozygosity

[d] Gene diversity

Table 3.3. Results of the linear regressions for the effects of network position (river distance) and straight-line distance on average genetic diversity metrics per site for *R. aniarum*. Gene diversity (H_S) is arcsine square root transformed prior to use in the correlation analysis.

	River Distance		Straight-line Distance	
	R^2	*p*	R^2	*p*
Gene Diversity (H_S)	0.001	0.900	0.002	0.88
Allelic Richness	0.5508	0.003	0.49	0.007

Table 4.3. Results of the linear regressions for the effects of network position (river distance) and straight-line distance on average genetic diversity metrics per site for *R. aniarum* when three outlier collection locations (two tributary sites and Freese Dam) are removed from the analysis. Gene diversity (H_S) is arcsine square root transformed prior to use in the correlation analysis.

	River Distance		Straight-line Distance	
	R^2	*p*	R^2	*p*
Gene Diversity (H_S)	0.1808	0.216	0.1857	0.213
Allelic Richness	0.87	**0.00008**	0.8656	**0.00009**

Table 5.3. Pairwise measures of F_{ST} based on 11 microsatellite markers among collection sites. Significance values given above the diagonal using the Weir and Cockerham theta estimate (1984), 10,000 permutations. Highlighted significance values shown to emphasize sites with significant pairwise comparisons among all other sites.

	Hwy 277	**Elm Creek**	**Hwy 83**	**Potter Falls**	**12 Mile Bridge**	**Freese Dam**	**Fishing Hole**	**Hwy 283**	**River Bend Ranch**	**FM 380**	**Heatchen Ranch**	**Lipan Creek**	**Paint Rock**
Hwy 277		0.0136	0.0109	0.0002	0.0052	0.0001	0.0006	0.0211	0.0045	0.0001	0.0012	0.0060	0.0077
Elm Creek	0.0065		0.0775	0.0011	0.0458	0.0001	0.0063	0.0662	0.0348	0.0003	0.0667	0.0290	0.0076
Hwy 83	0.0069	0.0038		0.4579	0.3096	0.0001	0.0125	0.0369	0.0365	0.0001	0.0218	0.0176	0.0041
Potter Falls	0.0117	0.0097	0.0000		0.0528	0.0001	0.0001	0.0006	0.0004	0.0001	0.0001	0.0004	0.0001
12 Mile Bridge	0.0089	0.0048	0.0011	0.0045		0.0001	0.0009	0.0655	0.0581	0.0001	0.0435	0.0306	0.0059
Freese Dam	0.0222	0.0105	0.0179	0.0240	0.0193		0.0001	0.0001	0.0001	0.0001	0.0003	0.0001	0.0001
Fishing Hole	0.0127	0.0084	0.0078	0.0166	0.0134	0.0166		0.0022	0.0004	0.0002	0.0169	0.0019	0.0001
Hwy 283	0.0056	0.0037	0.0050	0.0099	0.0042	0.0124	0.0104		0.4510	0.0002	0.0569	0.0046	0.1991
River Bend Ranch	0.0069	0.0044	0.0046	0.0095	0.0040	0.0180	0.0125	0.0000		0.0001	0.0303	0.0585	0.1133
FM 380	0.0313	0.0162	0.0274	0.0345	0.0315	0.0242	0.0204	0.0187	0.0172		0.1936	0.0002	0.0002
Heatchen Ranch	0.0122	0.0050	0.0077	0.0178	0.0062	0.0137	0.0086	0.0051	0.0062	0.0037		0.0618	0.1730
Lipan Creek	0.0105	0.0071	0.0085	0.0144	0.0071	0.0214	0.0139	0.0102	0.0051	0.0242	0.0066		0.0008
Paint Rock	0.0056	0.0055	0.0066	0.0130	0.0064	0.0192	0.0115	0.0013	0.0020	0.0160	0.0023	0.0118	

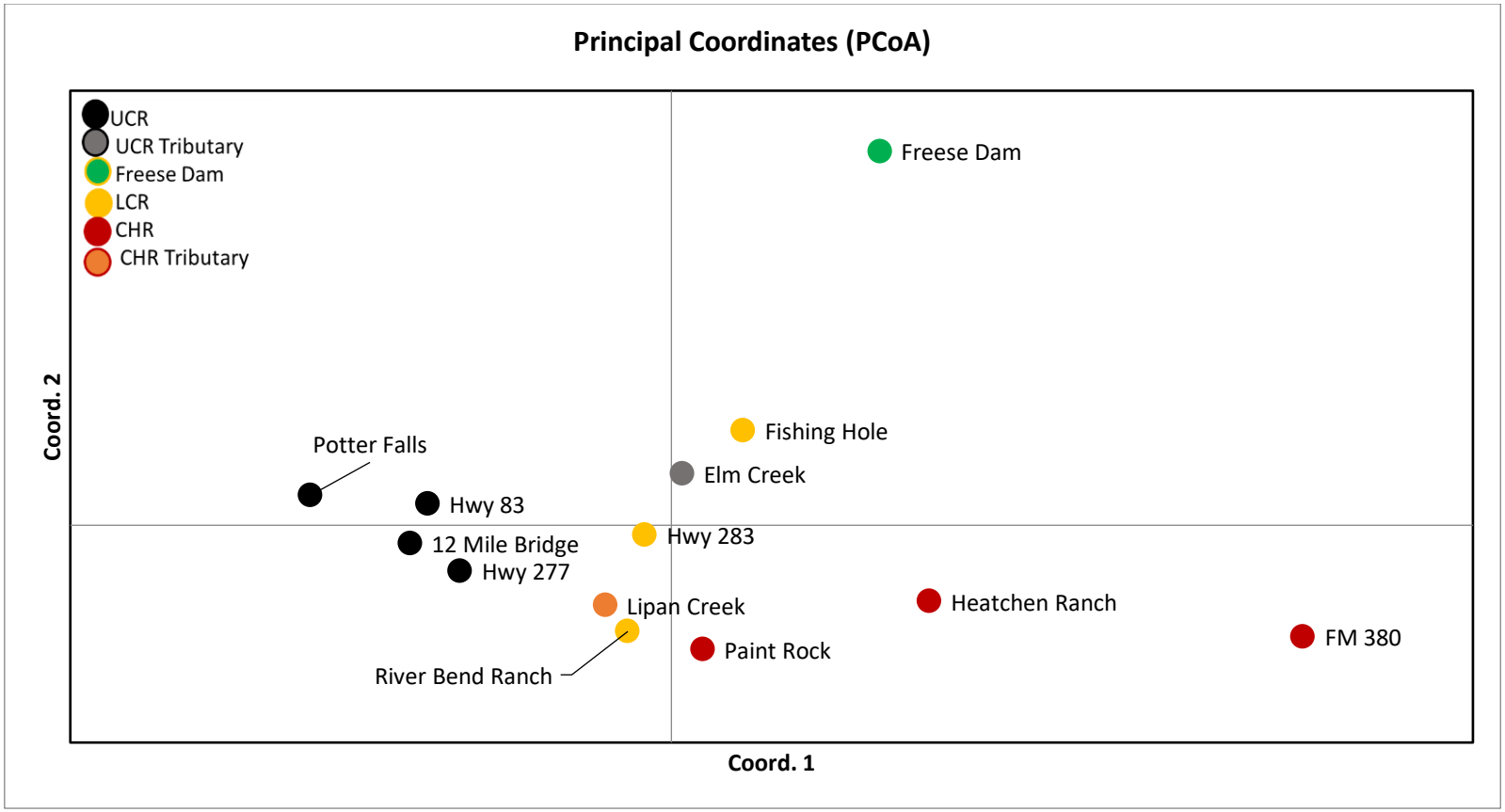


Figure 2.3. The PCoA shown for axis 1 vs. 2 (57.86% of the total variance) for the pairwise F_{ST} values among collection sites. Note that Freese Dam falls out separately from all other groups in the PCoA analysis but is geographically located immediately below the damn, near the historic confluence of the Colorado and Concho Rivers.

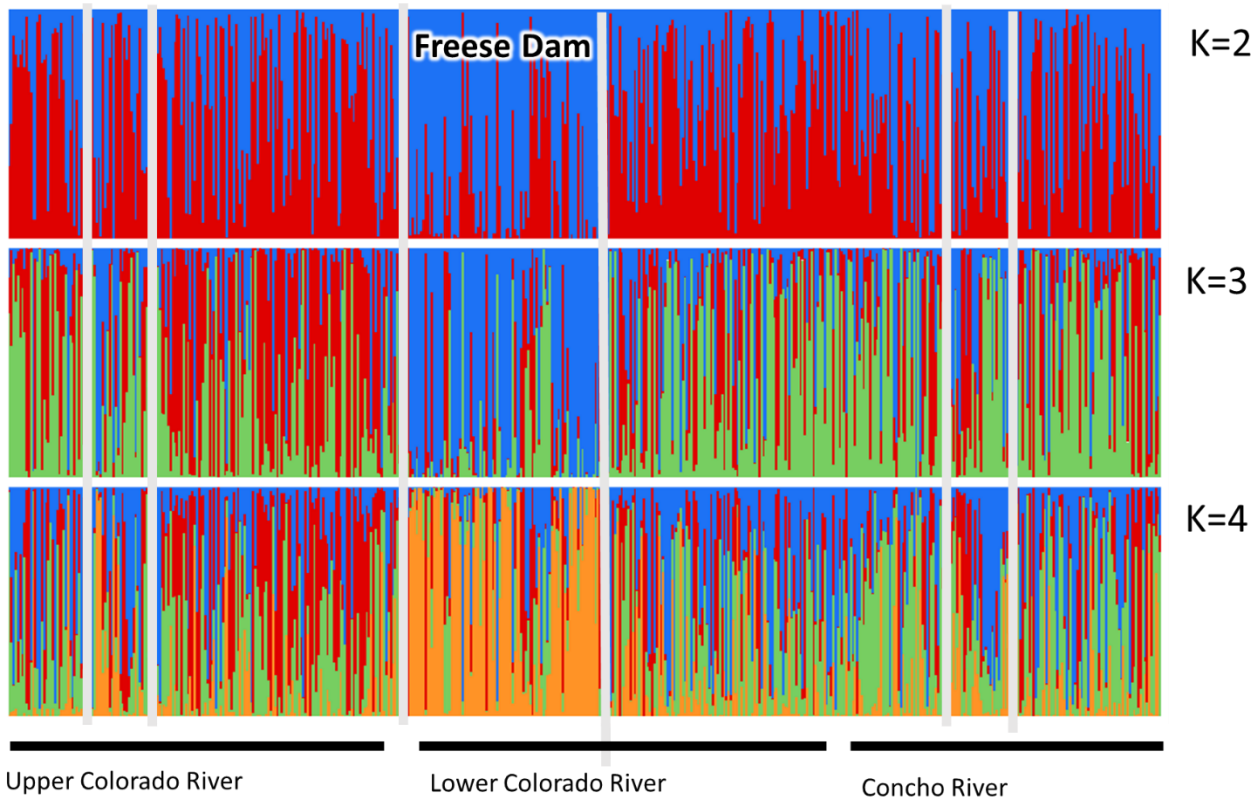


Figure 3.3. Results of the Bayesian clustering analysis in STRUCTURE for 529 *R. aniarum* individuals from the Colorado and Concho Rivers. Individuals are arranged by collection location from most upstream site on each of the three reaches of the river. Individuals collected from tributary collection sites are indicated on the plot but show no visible clustering compared to main-stem populations. Individuals within Freese Dam are dominated by a single genetic cluster from K=2. No additional structure is visible at each subsequent K values. All other populations show no distinct clusters among site or among the three reaches of the river.

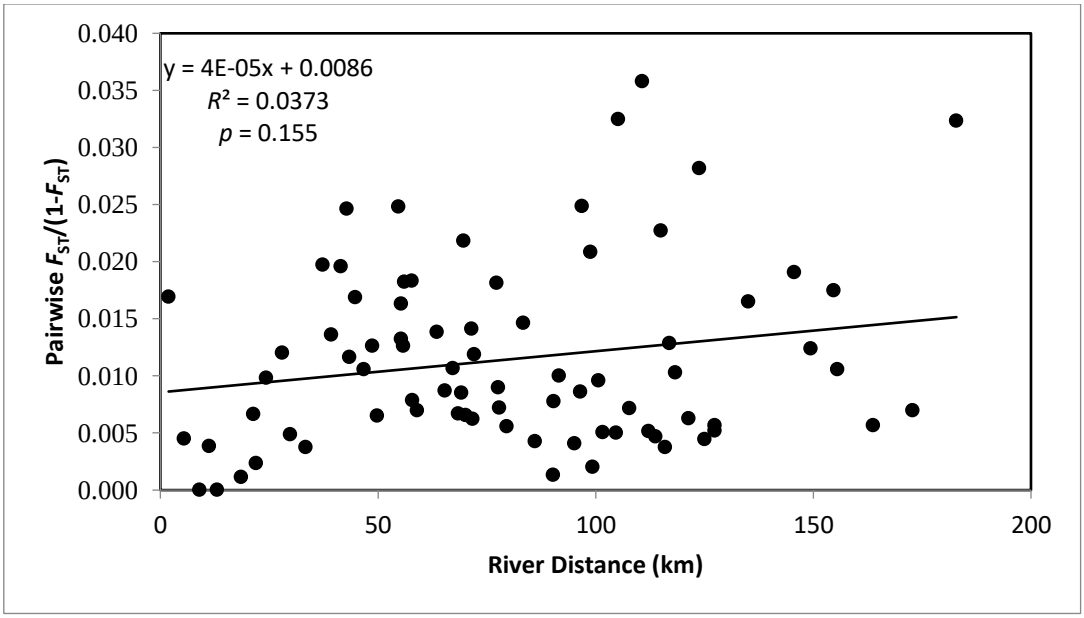


Figure 4.3. Results of the Mantel test for isolation by distance between pairwise F_{ST} (F_{ST}/ (1-F_{ST})) (Rousset 1997) for all main-stem and tributary sites on both the Colorado and Concho Rivers as a measure of genetic differentiation among sites and river distance.

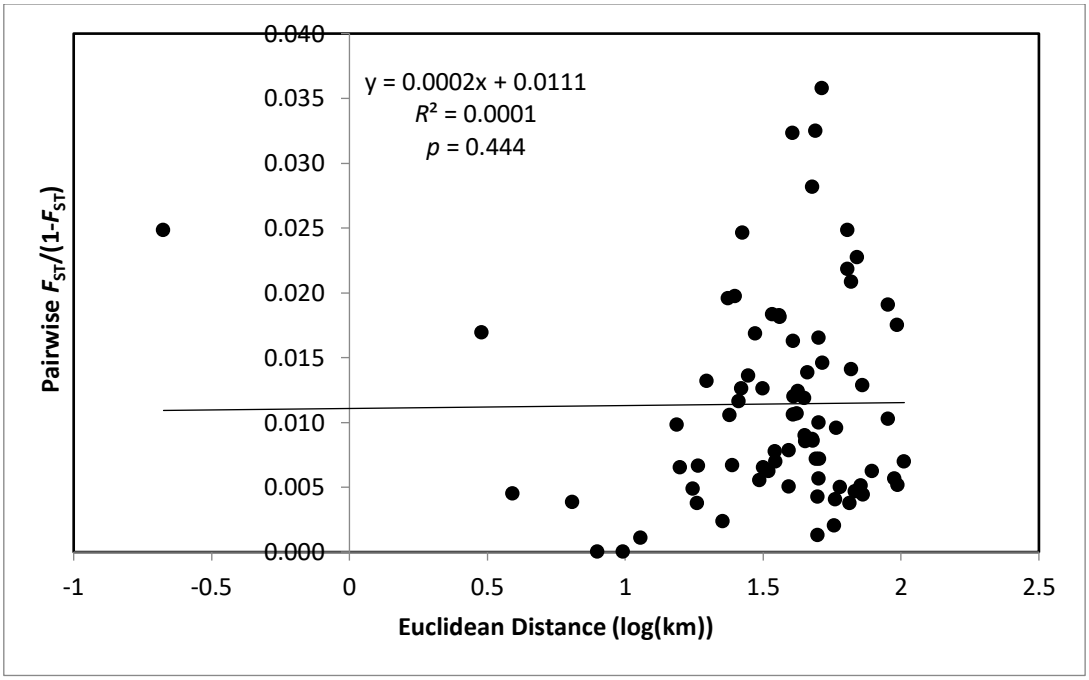
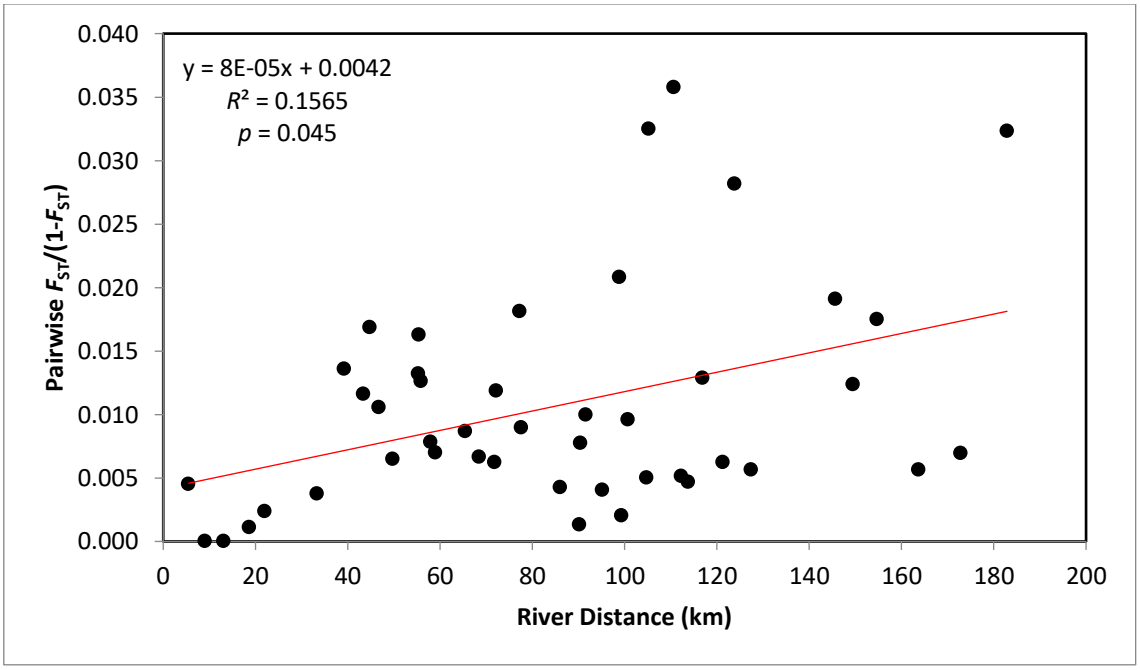


Figure 5.3 Results of the Mantel test for isolation by distance between linear pairwise F_{ST} (F_{ST}/ (1-F_{ST})) (Rousset 1997) and straight-line distance all main-stem and tributary sites on both the Colorado and Concho Rivers. Euclidean distances are log transformed based on the recommendation by Rousset (1997) for two dimensional habitats.



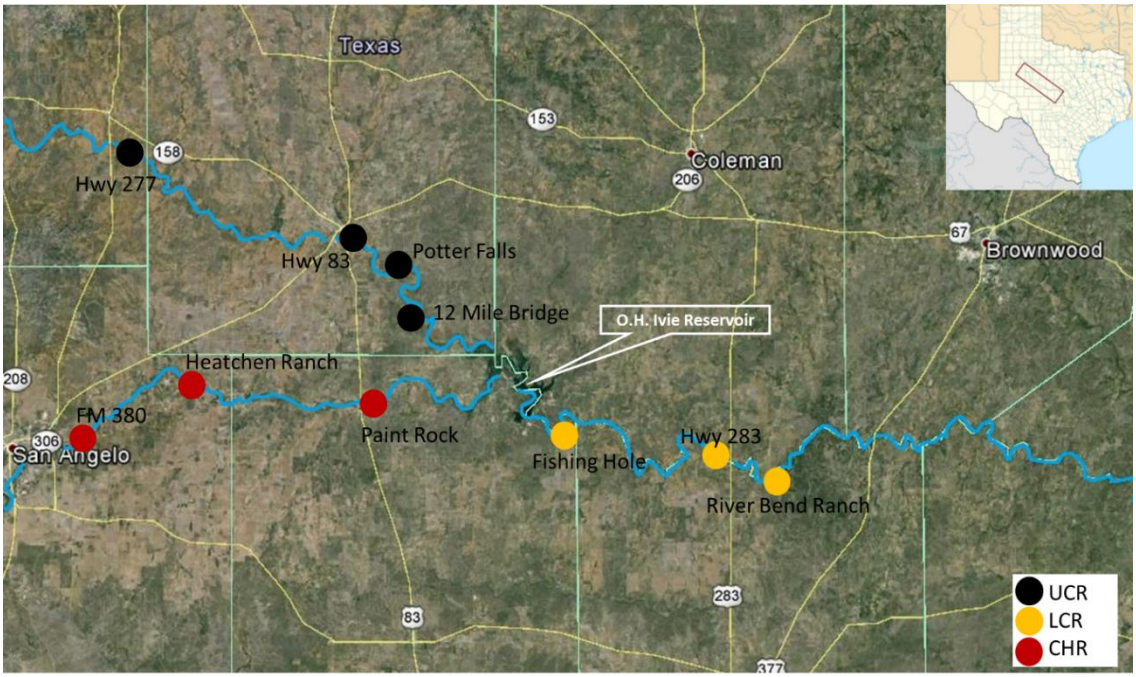


Figure 6.3. Results of the Mantel test for isolation by distance between pairwise F_{ST} ($F_{ST}/(1-F_{ST})$) (Rousset 1997) and river distance (km) as a measure of genetic differentiation among sites and river distance when the two tributary and Freese Dam collection sites are removed from the data set. (Google Earth Pro Images Landsat/Copernicus 2018).

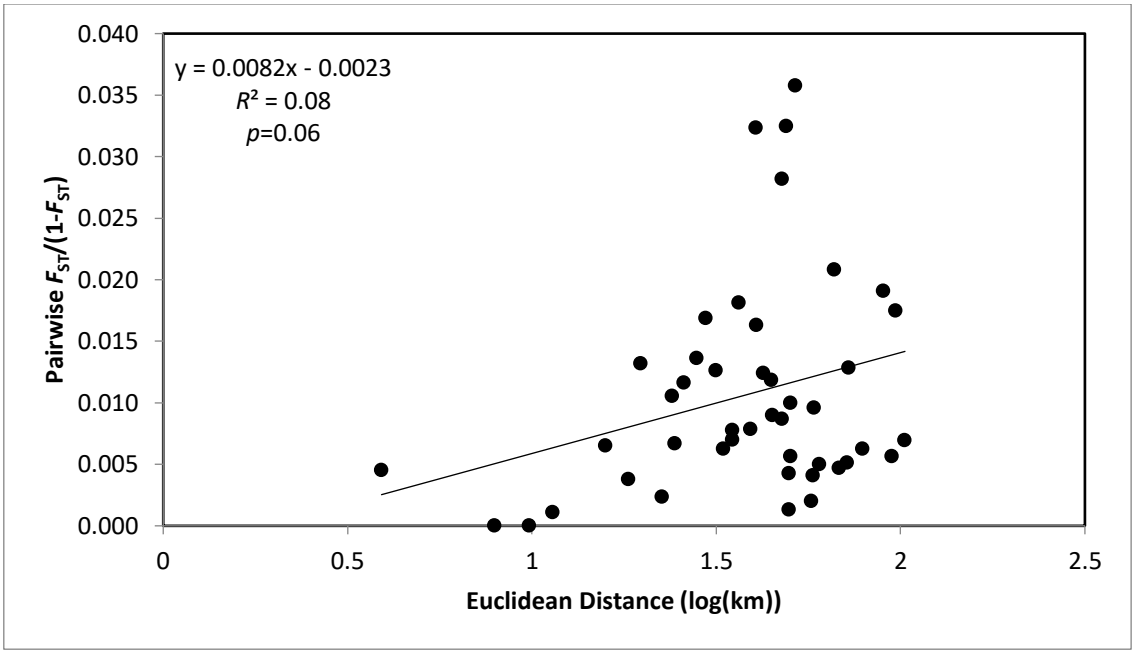
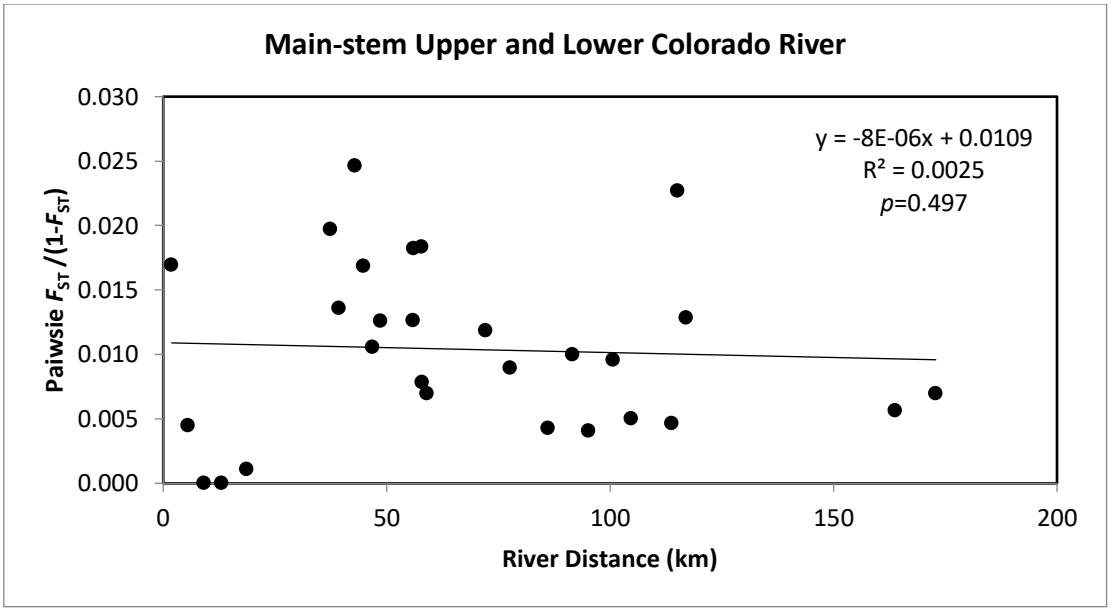


Figure 7.3. Results of the Mantel test for isolation by distance between pairwise F_{ST} (F_{ST}/(1-F_{ST})) (Rousset 1997) and straight-line distance for the 11 sites along the Colorado and Concho Rivers and two tributary locations. Euclidean distances are log transformed based on the recommendation by Rousset (1997) for two dimensional habitats.



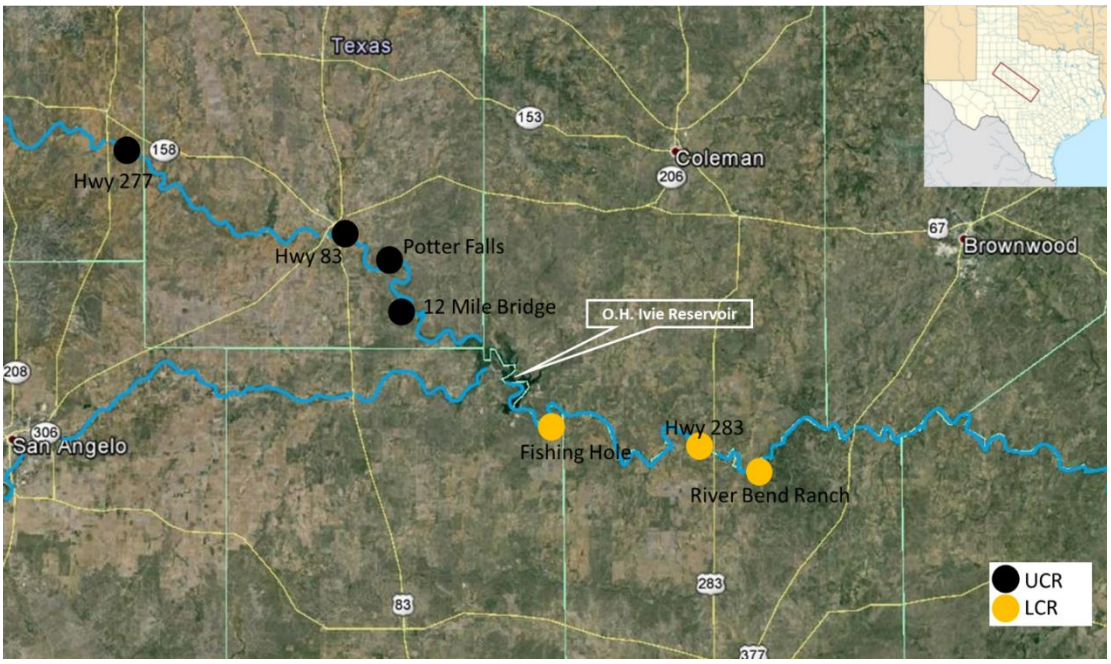
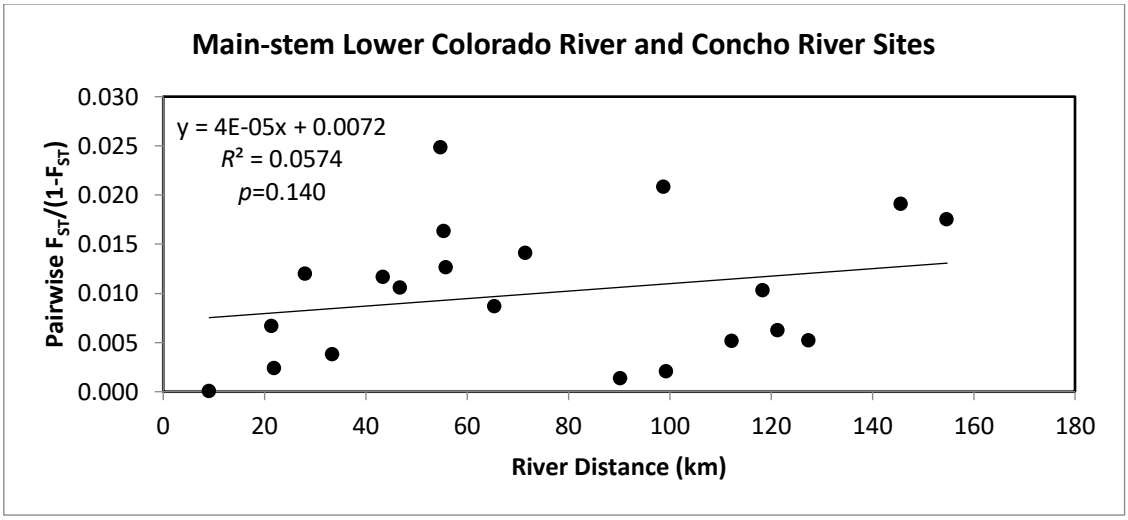


Figure 8.3. Results of the Mantel test for isolation by distance between pairwise F_{ST} (F_{ST}/(1-F_{ST})) (Rousset 1997) and river distance (km) as a measure of genetic differentiation among sites and river distance following an unbranched pathway from the upper Colorado River to the most lower Colorado River (excluding Freese Dam). (Google Earth Pro Images Landsat/Copernicus 2018).



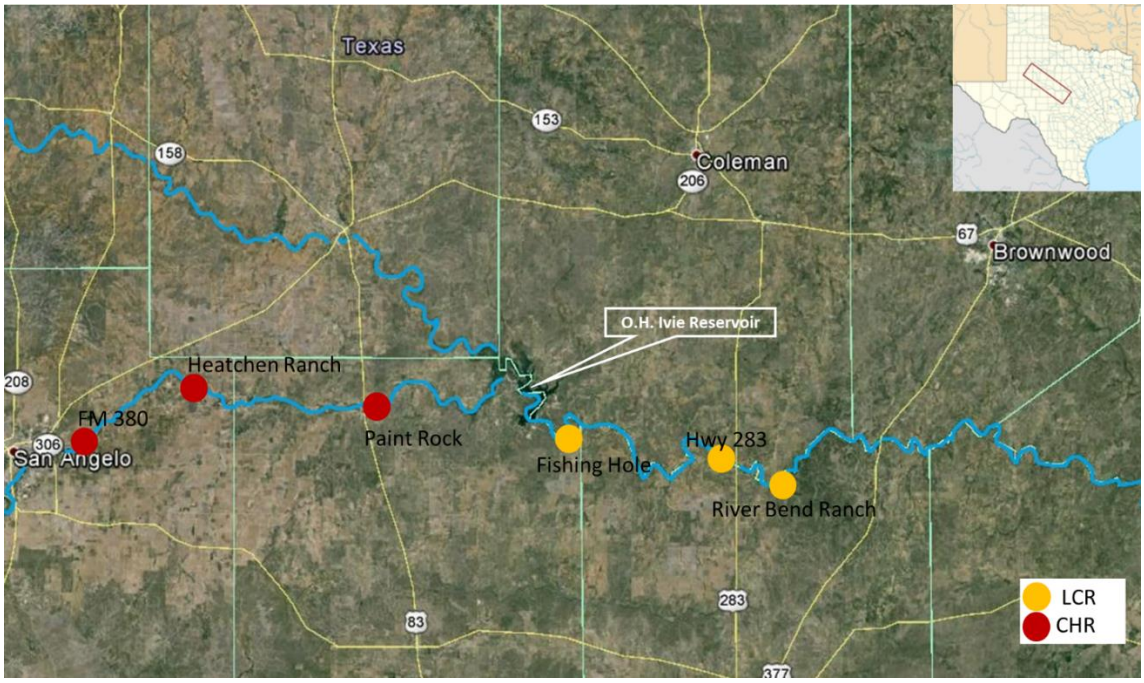


Figure 9.3. Results of the Mantel test for isolation by distance between pairwise F_{ST} (F_{ST}/(1-F_{ST}) (Rousset 1997) and river distance (km) as a measure of genetic differentiation among sites and river distance following an unbranched pathway from Concho River to the lower Colorado River (excluding Freese Dam). (Google Earth Pro Images Landsat/Copernicus 2018).

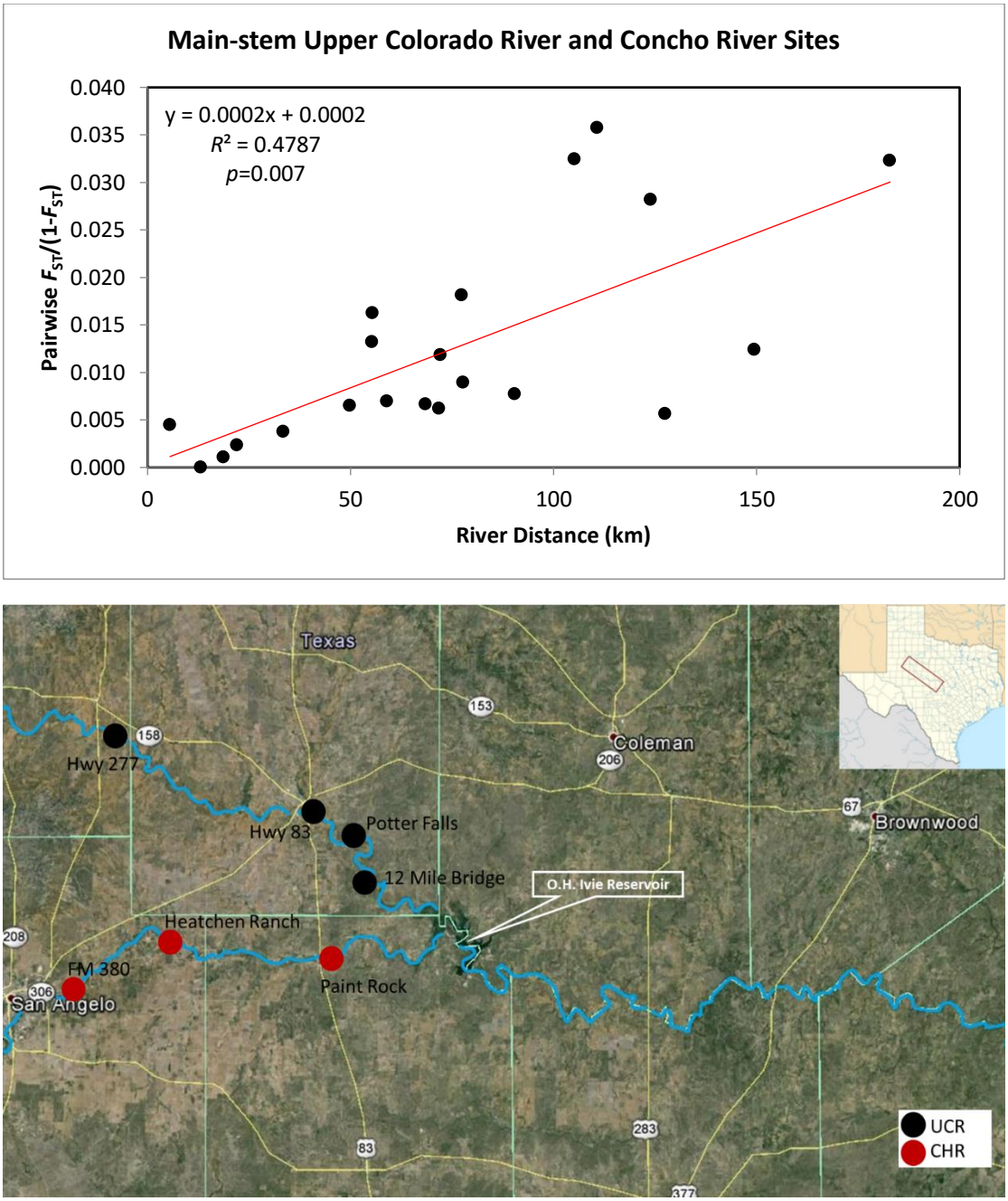


Figure10.3. Results of the Mantel test for isolation by distance between pairwise F_{ST} (F_{ST}/(1-F_{ST})) (Rousset 1997) and river distance (km) as a measure of genetic differentiation among sites and river distance following a branched pathway from Upper Colorado River (excluding Freese Dam) to Concho River. (Google Earth Pro Images Landsat/Copernicus 2018).

Discussion

Renifer aniarum exhibited high levels of gene diversity that were not significantly different among sites or among the three reaches of the river. There was a non-significant correlation between gene diversity and distance downstream. Allelic richness, while not statistically different among sites or among the three reaches of the river, exhibited a strong negative correlation with distance from the most downstream location. Allelic richness values in upstream sites were 9.52 on the Colorado River and 9.6 on FM 380, while allelic richness at the most downstream location was 10.904, a net increase in allelic richness of 1.34 alleles for the most downstream site. The correlation between allelic richness and distance downstream was more significant when the two tributary locations and the Freese Dam collection site were excluded from the analysis. The Mantel test for isolation by distance between sites was also not significant for either straight-line distance or river distance when all collection sites were included in the analysis. There was also no significant isolation by distance following non-branching routes from either the upper Colorado to the lower Colorado River, or from the Concho River to the lower Colorado River. However, there was significant isolation by distance when both upstream main stem branches and the lower Colorado River were included in the analysis, indicating that the isolation by distance pattern was driven by site placement on distal network branches.

When the entire data set is considered or when non-branching main-stem network pathways are considered, our results are largely consistent with the hypothesis proposed by Balsco Cost et al. (2012) who posited that parasites infecting hosts with

high dispersal abilities will not exhibit the patterns of genetic diversity predicted for organism's whose dispersal ability is strongly influenced by unidirectional stream drift. When considered alone, the larger spatial scale of the present study (172 km) compared to the 70 km distances did not appear to alter lack of isolation by distance. However, the additional complexity of river branches alters the pattern of isolation by distance and may account for the significant increase in allelic richness in downstream sites below the confluence of the two upstream reaches. The apparently unique local dynamics of the tributary sites and the population immediately downstream from Freese Dam also suggest that additional more local factors such as variation in connectivity between tributary and mainstream locations and human habitat alteration may contribute to patterns of isolation by distance and genetic diversity within river systems.

The low F_{ST} values for all pairwise comparisons combined with the lack of population subdivision among branches detected in the STRUCTURE analysis suggest that the DEN of the Colorado and the Concho River does not create population subdivision correlated with branches. Rather, the DEN caused isolation by distance patterns between populations located on upstream branches. Of the three potential definitive hosts for *R. aniarum* on the Colorado and Concho Rivers, *N. erythrogaster* and *N. rhombifer* had higher prevalence and intensity of infection than *N. paucimaculata*. Lack of strong population subdivision among the river branches and low F_{ST} values between sites for *R. aniarum* may be concordant with the dispersal abilities of *R. aniarum'*s mobile definitive host, *N. erythrogaster*, which has been reported to exhibit no population subdivision between the three reaches of the Colorado

and Concho Rivers (Rodriguez et al. 2012). However, this assessment was based on 8 *N. erythrogaster* individuals from the Colorado River and 8 from the Concho River using 5 microsatellite markers. The combination of few individuals sampled and few microsatellites may not have been sufficient to determine fine-scale patterns in *N. erythrogaster* population structure and its concordance with *R. aniarum* population structure. The population structure of *N. rhombifer* along the Colorado and Concho Rivers is currently unknown but the species is common and widespread. The population structure of the intermediate host species, (*Physa* snails and *Lithobates spp,* and. tadpoles) for *R. aniarum* are also currently unknown. Whelan et al. (2019) found strong support for both unidirectional stream drift and dendritic river architecture on the population structure of the Round Rocksnail (*Leptoxis ampla*) while other authors have emphasized strong patterns of upstream migration and lack of population subdivision in other freshwater gastropods (Kappes and Hasse 2012). Tadpole migration upstream is likely to be limited, but if infective *R. aniarum* metacercariae are retained in adult frogs, it is possible they too can contribute to *R. aniarum* population structure through net upstream migration (Funk et al. 2005). Future research on this system could include a co-structure analysis between *R. aniarum* and the population structure of the definitive and intermediate host species to determine which host may be driving this pattern.

Freshwater autogenic parasites, parasites whose life cycles are fully aquatic in invertebrates, fish and/and or amphibians, are considered "locally" transmitted and often exhibit patterns of population constrained by river network (Blasco-Costa et al. 2013; Criscione and Blouin 2004). Pettersen et al. (2015) found significant patterns of

population structure and a high degree of endemism associated with network placement for *G. thymalli* within a single drainage basin, the Glomma River system. *Gyrodactylus thymalli* is a monogene parasite that infects fish and is spread through direct contact between individuals, while the presence of waterfalls and dams present barriers to upstream Grayling dispersal which in turn prohibited the dispersal of *G. thymalli.* Criscione et al. (2007) found strong support for differentiation of *Plagioporous shawi*, a fully aquatic trematode infecting salmonid hosts, among three different river drainages. Allogeneic parasites, parasites whose definitive host may be a bird or a mammal have been shown to exhibit no population substructure, even over large geographic scales (Blasco-Costa and Poulin 2013). Louhi et al. (2010) found no population subdivision in the trematode parasite *Diplostomum pseudospathaceum* whose definitive host is a bird, in populations separated by over 300 km. *Schistosoma mansoni*, which can infect mammals as a definitive host exhibited population structure but no pattern of isolation by distance among five sites on Guadeloupe Island (Prugnolle et al. 2005). While *R. aniarum* populations did not exhibit strong population structure among branches, *R. aniarum* gene flow is not uniform within network space, as there were some differences in population connectively based on site placement within the river network and significant patterns of isolation by distance for distal main-stem river branches. The most upstream location on the Colorado (Hwy 277) was significantly diverged from all of the main-stem populations on the Concho River. Similarly, the most upstream location on the Concho River (FM 380) had significant levels of population subdivision in comparison to all other locations on the Concho and Colorado Rivers. Both of these

patterns are likely driven by the more distal placement of these sampling sites in comparison to sites located more centrally within the network. The results of the PCoA reveal that levels of population subdivision are lowest among sites on the upper Colorado River, which formed the most discreet cluster. Sites from the Concho River fell out within the bottom right quadrat, separate from all sites on the Upper Colorado River. The lower Colorado River sites were located between the two UCR and CHR clusters, indicating higher levels of gene flow between the lower Colorado River and Upper Colorado River and between the lower Colorado River and Concho River than between the Upper Colorado River and the Concho River. The tributary populations clustered with the main stem lower Colorado River sites rather than sites located on geographically adjacent locations on the upper Colorado and Concho Rivers, suggesting that distinct local patterns of transmission may be affecting the tributary locations. Thus, patterns of population structure in *R. aniarum* appear to be intermediate, neither highly structured among river branches as might be predicted for an autogentic parasites species, nor fully panmictic, as expected for an allogenic species.

Renifer aniarum individuals collected from the Freese Dam site were consistently diverged from all other sampling locations and exhibited greater number of pairs of loci in linkage disequilibrium compared to other collection sites. Freese Dam is located immediately downstream from O.H. Ivie reservoir, near the confluence of the Colorado and Concho Rivers and so potential causes of this pattern are currently unclear. Atkinson and Bartholomew (2010) studied the population structure of the myxozoan parasite *Ceratomyxa shasta* in several species of salmonid fish in the Kalmath River

using the mitochondrial ITS-1 marker. Dams have separated upper, middle and lower basins of the river for approximately 80 years. Atkinson and Bartholomew (2010) found the population structure of *Ceratomyxa shasta*) (Myxozoa) was highly structured spatially between reaches of the river separated by dams and between host species. Distinct *C. shasta* genotypes were restricted to river basins isolated between dams, indicating that the dam was likely responsible for the isolation of the parasite between reaches. Pettersen et al. (2015) also state that the presence of reservoirs act as a barrier to host-parasite dispersal with their river network but did not address the length of time these barriers have been in place. Construction was completed on O.H. Ivie reservoir in 1989, a duration of 30 years previous to the current date. Our results did not find evidence of long-term disruptions in population connectivity between other *R. aniarum* populations. However it is possible the presence of O.H Ivie reservoir may have impacted *R. aniarum* transmission in other ways. The presence of dams has been documented to significantly alter the transmission of human blood flukes, *Schistosoma mansoni*, by reducing salinity and thus enabling colonization by the freshwater snail host, *Biomphalaria pfeifferi* (Campbell et al. 2010). Diakite et al. (2017) reported increases in snail abundance and changes in community composition in response the construction of new dams due to changes in habitat stability from flood control. Increased habitat predictability can also alter snail life history characteristics such as fecundity and survivorship, characteristics that can also influence trematode transmission (Diamond 1982; Charbonnel et al. 2002). Characteristics of the river immediately below O.H. Ivie reservoir are distinct from other locations along the

Colorado and Concho Rivers, with dense aquatic vegetation and wide marshy areas adjacent to the river bed and significant accumulation of fine-particle silt, suggesting greater habitat stability at this collection site. It is possible that the habitat alteration may have in turn altered transmission between the first and or second intermediate host, changes in intermediate host density or community composition. The high levels of linkage disequilibrium in the Freese Dam population may also suggest an admixed parasite population below the reservoir in the newly created stabile habitat. It would be of interest to determine if snail community composition differ among collection sites. As this study did not address first or second intermediate host densities, population dynamics or community composition, the exact cause of the unusual parasite population structure below Freese Dam remains unclear. Additional research is required to elucidate what may be driving this pattern of population subdivision below O.H. Ivie reservoir.

Conclusions

Parasites are ubiquitous and important components river habitats but ecological factors that shape parasite population structure within rivers are largely unexplored. The results of this study confirm Basco-Costa et al.'s (2012) assertion that the population structure of parasites that infect hosts with high dispersal abilities will not be strongly influenced by unidirectional stream drift by facilitating upstream parasite dispersal for non-branching river pathways. Similarly *R. aniarum* host dispersal also appeared to largely negate the potentially isolating physical characteristics imposed by the network architecture of the Colorado and Concho Rivers, however, unlike fully-autogenetic

parasites, gene flow was not uniform across all collection sites and patterns of isolation by distance were observed between network branches. Local patterns of transmission within tributaries and below O.H. Ivie reservoir suggest variation in connectivity and human alteration can also impact *R. aniarum* population structure within the river system. Thus *R. aniarum* transmission and population structure appears to be the result a more complex interaction between host dispersal, local transmission dynamics and habitat structure. Additional compounding factors such as differences in riverscape permeability to host dispersal, potential terrestrial dispersal, and variation in connectivity during long periods of drought and flash-flooding events that characterize the semi-arid habitat of the Colorado and Concho Rivers are likely contributing to the variation in connectivity among *R. aniarum* populations.

CHAPTER IV

CONSERVATION GENETICS OF THE CONCHO WATER SNAKE (*Nerodia hateri paucimaculata*)

Introduction

The Concho water snake (*Nerodia harteri paucimaculata*) is a relatively small natricine snake endemic to the Concho and Colorado rivers of central Texas (Figure 1.4) (Scott et al. 1989). It has one of the most restricted ranges of any snake in the United States, occupying sections of the Colorado River, the Concho River downstream of San Angelo, Texas and reservoir shoreline in Spence, O.H. Ivie and Ballinger Lake. The habitat of *N. h. paucimaculata* is characterized by riffles and rocky shores where both adults and juveniles prey on small minnows (Cyprinidae) within the riffle (Greene et al. 1994; Scott Jr. et al. 1989). Adults have also been observed in deeper pools (Whiting et al. 1997). In reservoirs, Concho water snakes occupy rocky shoreline with shallow slopes and low wave action but have also been found in rocky cliffs that end abruptly at the water (Whiting et al. 1997).

Several authors have noted that the persistence of Concho water snakes in newly created lake and reservoir habitat is an indication that the Concho water snake may not be as specialized in their habitat as was once believed (Greene et al. 1994; Scott et al. 1989; Whiting et al. 1997; Whiting et al. 2008). Despite its restricted geographic range, Tinkle and Conant (1961) noted in their description of the Concho water snake that populations occur at high densities within suitable habitat, with 114 individuals collected

from a single location on the Upper Colorado River just south of Robert Lee, Texas in only 3 days. Subsequent authors noted similarly abundant populations, with 236 individuals captured in Spence Reservoir in 1990-1992 (Whiting et al. 1998). Concho water snakes are rarely encountered more than a meter from the water because of their habitat affinity and perhaps due to their small size and potentially higher rates of evaporative water loss (Scott et al. 1989; Winne et al. 2001). They are thought to have a reduced dispersal capacity, moving longer distances (4 km) only in response to habitat loss (Whiting et al. 1997). The combination of restricted range, habitat specialization, high site fidelity and reduced dispersal capability make Concho water snakes potentially vulnerable to habitat loss and degradation.

The Colorado and Concho Rivers are the primary source of water for several municipalities in semi-arid west Texas and provide an important source of the irrigation for crops and livestock. E.V. Spence Reservoir was completed in 1969 on the Upper Colorado River within the northern most extremes of the Concho water snake's range. Along the Concho River, Lake Nasworthy, O.C. Fisher Reservoir, and Twin Buttes Reservoir, all located near San Angelo and completed in 1930, 1952 and 1963 respectively, constrict the flow of all three forks of the Concho River, Spring Creek, and Dove Creek upstream from their confluences with the primary Concho River near San Angelo, TX. Concho water snakes have been extirpated upstream of San Angelo although a small population was still located at the Bell Street Bridge around 2008 (Scott et al. 1989, USFWS 2011).

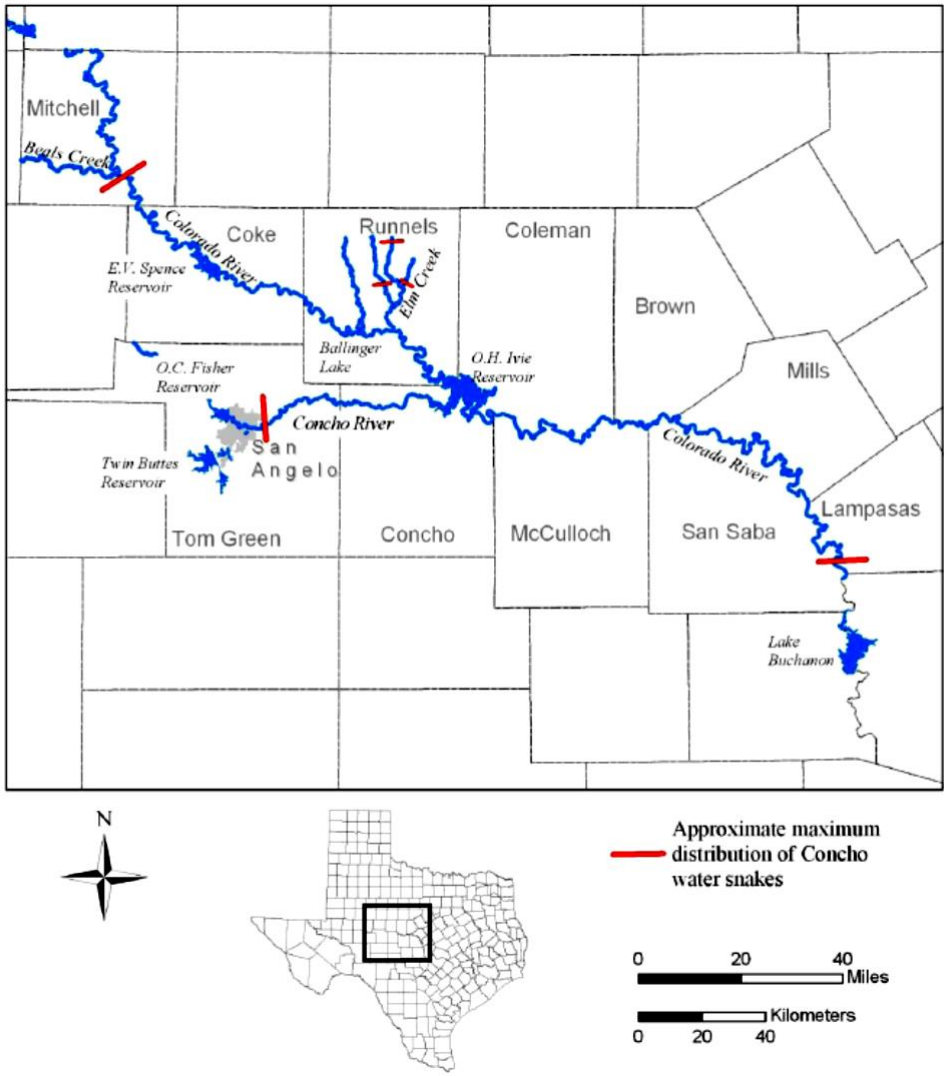


Figure 1.4. Estimated range of the Concho water snake at the time of de-listing (USFWS 2011). This species has previously been detected between E.V. Spence Reservoir and three miles downstream of the Hwy 377 crossing of the Colorado River south of Brownwood, TX. Populations on the Concho River were previously recorded between the Bell Street Bridge Crossing in San Angelo, TX to the confluence of the Concho and Colorado Rivers in O.H. Ivie Reservoir.

The construction of O.H. Ivie Reservoir at the confluence of the Colorado and Concho Rivers within the core range prompted the USFWS to list the Concho water snake as Threatened and the state of Texas to list it as endangered in 1986. Construction of O.H. Ivie Reservoir was completed in 1989, inundating approximately 25% of the central habitat range of the snake and potentially creating three disconnected populations: 1.) Colorado River populations downstream of O.H Ivie reservoir, 2). Colorado populations upstream of O.H. Ivie Reservoir and 3). Concho River populations upstream of O.H. Ivie Reservoir (Whiting et al. 2008). Extensive field work in the late 1980s and early 1990s to determine the effects of dams on the snake, specifically the construction of O. H. Ivie reservoir suggested that the Concho water snake was not restricted to the riffle sections but that they would also use habitats within lakes, especially areas with rocks along the shoreline (Greene et al., 1994; 1999; Whiting et al., 1997; 2008). Based on 10 years of intermittent survey data, Whiting et al. (2008) found the Concho water snake was able to persist in Lake Ballinger, E.V. Spence and O.H. Ivie reservoirs at low densities. Additional surveys throughout the range of the snake prompted the US Fish and Wildlife Service to remove the Concho water snake from the federal endangered species list in 2011 (Federal Register 76:66780-66804; July 8, 2011). However estimates of population size, viability, and survival rates could not be determined due to low sample sizes and inconsistency in survey effort between sampling years.

The Endangered Species Act section 4(g)(1) requires that the Unites States Fish and Wildlife Service, in conjunction with the state agencies conduct at least 5 year of

population monitoring following delisting to ensure the status of the species does not change. This study was undertaken to fulfill in part the requirements of the first three years of the post-delisting monitoring plan required by the USFWS 15 year monitoring plan. However there are inherent difficulties associated with monitoring snake populations. Snakes are widely accepted as among the most difficult vertebrate groups to study owing to their short activity periods, cryptic coloration and secretive behavior (Durso et al. 2011; Willson et al. 2011). Previous estimates of detection probabilities of water snakes ranged from 0.03 to 0.46, with considerable interspecific variability and not accounting for stochastic environmental variation in temperature and precipitation which can further repress snake detectability (Durso et al. 2011). These factors were often compounded for snake species that inhabit inaccessible aquatic or fossorial habitat or are largely confined to privately-owned lands. There are inherent difficulties in sampling the Concho water snake such as temporal variation in suitable habitat and subsequent shifts in snake occupancy and dramatic reductions in detectability of Concho water snake during periods of drought. As each of these factors are likely to continue to impact future monitoring efforts, it is especially important to provide managers with additional monitoring tools.

Rodriguez et al. (2012) were the first to study the population genetics of snakes in the Colorado and Concho River using mitochondrial (Cyt-*b*) and 5 microsatellite markers originally developed for *N. sipedon*. They collected 30 and 34 Concho water snakes from the Concho and Colorado Rivers, respectively. They reported low levels of mitochondrial haplotype diversity and evidence of population structure between the

Concho and the Colorado Rivers in the Concho water snake in both the mitochondrial and microsatellite markers. They also found evidence of a potential recent bottleneck for samples collected from the Concho River. I revisited the system as part Phase I monitoring requirements of the post-delisting monitoring plan (USFWS 2011).

This study presents the results of the first 3 years of the required post-delisting monitoring of the Concho water snake. This investigation had three primary objectives: 1). To develop species-specific microsatellite markers for population genetics analysis 2). Describe the genetic diversity and current population structure of the Concho water snake and 3). Estimate the current effective population size. The results of this study will serve as a baseline estimate for comparison during Phases II and III of the post-delisting monitoring of the Concho water snake and provide an important set of tools for future efforts in the long-term conservation of *N. h. paucimaculata*. I will also discuss the conservation implications of the results from the initial monitoring efforts and potential questions that may be important in future efforts in the long-term conservation of *N. h. paucimaculata.*

Methods

Surveys and Sample Collection

Surveys were conducted at 19 post delisting monitoring sites spanning the range of the Concho water snake from 2013 to 2015 (Figure 2.4). Snakes were captured through the use of partially submerged minnow traps or captured by hand during active searches. A total of 109 Concho water snakes were captured at seven of the 19 sites. Sites where Concho water snakes were collected were centered on riffles. Multiple

individuals were captured at each riffle site, except for Hwy 277 and Potter Falls, where only one individual was captured from the riffle. Tissue samples were collected in the form of small caudal scale clips and stored in 70% EtOH prior to extraction. Despite extensive sampling, no Concho water snakes were encountered on the Concho River. The 2013 and 2014 surveys were conducted during extreme drought conditions (USGS Annual Water Reports 2016). Mean annual stream flow in the upper Colorado near Hwy 277 was 0.015 ft^3/sec and 0.031ft^3/sec in 2013 and 2014. Mean annual stream flow increased to 0.73 ft^3/sec in 2015. The 2015 spring and summer sampling season experienced the largest influx of rain that west-central Texas had experienced since the Concho water snake post-delisting monitoring was initiated in 2013, with mean annual stream flows increasing at all USGS monitoring sites on the Colorado and Concho Rivers. Despite the heavy rains during this time, the sites on the upper Colorado River upstream of Hwy 277 South of Bronte TX did not have water in the river bed.

DNA Extraction and Microsatellite Development

In 2013, a tissue sample collected from the Hwy 277 survey site in 2013 was submitted to the Sequencing and Genotyping Facility at the Cornell Life Sciences Core Laboratory Center (Ithaca, NY) in order to develop a microsatellite library following protocols described in Nali et al. (2014). A subset of the resulting library of potential primers and loci were selected in order to encompass variation in product size, repeat count, and dimeric, trimeric and tetrameric repeats. The M13 method for genotyping was used by attaching a 20-bp tag to the 5' end of the forward primer (Schulke 2000).

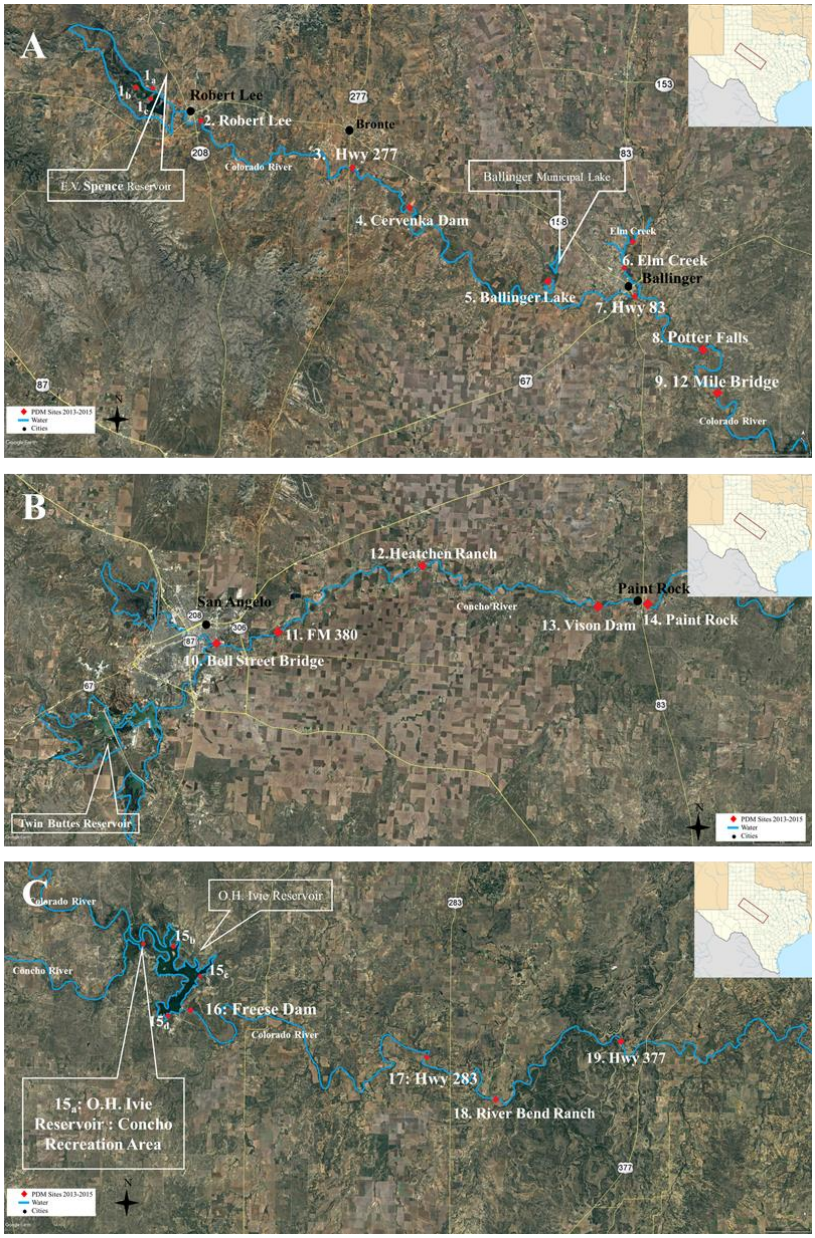


Figure 2.4. Post-delisting monitoring sites for *N. h. paucimaculata* along the upper Colorado River upstream of O.H. Ivie Reservoir (**A**), along the Concho River Upstream of O.H. Ivie Reservoir (**B**) and downstream from the confluence of the Conch and Colorado Rivers (**C**). Tissue samples were collected from 4 locations on the upper Colorado River (Hwy 277, Hwy 83, Potter Falls, and 12 Mile Bridge), 1 location within O.H. Ivie Reservoir (Concho Recreation Area) and two location on the lower Colorado River (Freese Dam and Hwy 283). (Google Earth Pro Images Landsat/Copernicus 2018).

A short tail sequence (GTTTCTT) was added to the 5'end of the reverse primer to reduce polyadenylation (Brownstein et al. 1996). DNA was extracted from tissue taken from small, ventral scale clips of 6 *N. h. paucimaculata* individuals using a 5% chelex and 0.2 mg/mL protienase K in 200 µL volume reaction. The samples were incubated at 56 ºC for 12 hours and then boiled at 100 ºC for 8 minutes. PCR amplification was performed in 10 µL reactions containing 3.1 µL ultrapure water, 5 µl 2X Qiagen Type-IT kit Master Mix, 0.16 µL fluorescent-labeled M13 primer (Applied Biosystems: FAM), 0.08 µL M13-labeled forward primer, 0.16 µL of 10 µM reverse primer and 1.5 µL of genomic DNA. The thermocycler profile was 94 ºC for 5 minutes, 31 cycles of 94 ºC for 30 seconds, 56 ºC for 45 seconds, 65 ºC for 45 seconds, followed by nine cycles of 94 ºC for 30 seconds, 53 ºC for 45 seconds 65 ºC for 45 seconds and extension at 65 ºC for 10 minutes. PCR-product was visualized on a 2% agarose gel run in 0.5X TBE buffer at 95 ºC for 45 minutes. Primers that yielded discrete bands in the expected product size range were sent to the DNA Analysis Facility on Science Hill at Yale University (Princeton, NJ, USA) and visualized on a 3730xl 96-capilary Genetic Analyzer with 500-LIZ size standard. Samples were genotyped on GeneMarker 2.6.4. Initially, I tested primer pairs for 48 loci in a subset of 6 Concho water snakes. Of these 48, 13 were found to amplify consistently and produced bands that could be scored reliably. I genotyped 109 Concho water snake individuals collected between the 2013 to 2015 sampling years at these 13 loci (Table 1.4). Five of the six cross-amplified microsatellite markers developed for other snake species and used in *N. h. paucimaculata* by Rodriguez et al. (2012) were also genotyped in this study: Nsu3,

Nsu4 (*Nerodia sipedon*, Prosser 1999), 3Ts (*Thamnophis sirtalis*, Garner et al. 2002), Ts3 (*T. sirtalis*, McCraken et al. 1999), and Ebou l (*Elaphe obsoleta*, Blouin-Demers and Gibbs 2003). Locus Nsu6 (Prosser et al. 1999) was not included in this study as it was found to be monomorphic in *N. h. paucimaculata* by Rodriguez et al. (2012). The M13 method for genotyping, shot-tail sequence tag, PCR amplification protocols, PCR product visualization and genotyping were used as described above for the loci developed in this study. Data from both the new microsatellite loci and the cross amplified loci were combined for use in subsequent analyses. Three data sets were created and analyzed: 1). Full data set with 109 individuals and 16 loci (13 novel loci, and 3 from Rodriguez et al. (2012). 2). Reduced data set of 92 individuals with sibling groups removed and 3). The full data set with 109 individuals and 5 loci from Rodriguez et al. (2012). Monomorphic loci from Rodriguez et al. (2012) were included only in the diversity estimates for comparative purposes.

Genetic Diversity and Equilibrium Tests

Sixteen loci amplified for the Concho water snake were included in calculations of gene diversity and equilibrium tests. Gene diversity (H_s), the number of alleles per locus (A_N), and allelic richness (A_R) (rarefied number to the lowest region sample size of N = 20) were calculated in FSTAT 2.39 (Goudet 2001). The number of private alleles was calculated using GENALEX 6.502 (Peakall and Smouse 2012). Estimates of F_{IS} (which quantifies the proportional change in heterozygosity due to deviations in Hardy Weinberg equilibrium) were conducted in in FSTAT 2.39 (Goudet 2001).

Table 1.4. Properties of the 13 *N. h. paucimaculata* microsatellite loci developed for use in this analysis

Primer Name	Primer Sequence (5' to 3')	Repeat Motif	T_a(°C)	Size Range (bp)
NePa 1470	F: TCATAAGAATCTGTTGTCTAGTCACAC	AGAT	56	289-313
	R: AATTGTTCACTGCCCAGAATCATAG			
NePa 5932	F:ACAACGACCTCTTTATGTAATGCAG	AAGT	56	330-342
	R: ACAAATGTATATTGCTGGTCTTTGAC			
NePa 12681	F: TATCAACCATTAGCACCAGTCAATG	AAC	56	196-208
	R: CAAGTTGAAATCCATAGCACGTTTC			
NePa 21288	F: TATGTCCATGCATTTCTTCCTCAAG	AAC	56	167-182
	R:CTGCCTGTCTGATCAATACCAAAC			
NePa 2349	F: AGTGGTAGAATTACACATCAACACC	ACAT	56	276-3517
	R: TTCTGACTATGACAACAATGGATTC			
NePa 9687	F:TCCGTTGAAGTAAGAGTTTCCAAAG	ACAT	56	278-286
	R:CAACAGTTCAGGAAGGTTTCAATTG			
NePa 1277	F: ATTGCTAGTCCATGTATGATATGCC	AAAC	56	350-362
	R: AATCATTCCCAACATTAGGCTCTTC			
NePa 2048	F:ATCTTAGCCACTGAGCCACTG	ACAT	56	350-358
	R:TGTCATATCACACAAACAGCAATTG			
NePa 2287	F: TGATCTTAACCAGTTCCAAGTTAGC	ACT	56	200-215
	R: TGTCTTCCTACATTCTCCCAGTTG			
NePa 11248	F:ATGGATGCAATGAACATGAATTTGG	AAC	56	260-265
	R:TGAATGTCTTGGCTGAATATCACTC			
NePa 12577	F:CTTGCTAAGTGATGTATTTGAATGATAC	ACT	56	230-254
	R:TCTTGGCAGATAGAACTTGTCAATG			
NePa 22153	F:TACATTCGGAGTATAAGACACACCC	ACAG	56	169-185
	R:ACTGGAGGTTGTTGATGGAATAATAG			
NePa 9972	F:AAATTTGATATCCCTTCAGTAGCCG	ACAG	56	317-321
	R:GGCCACAACAGGTAGGTAGG			

Tests for significant differences in diversity metrics and F_{IS} were carried out in FSTAT with 2000 randomizations. Genetic diversity estimates for the five Rodrigues loci were calculated as described above for comparative purposes.

Sibling Identification

Exploration of the data set for similarities in multilocus genotypes (MLG) revealed that a number of individuals had genotypes that differed at only one or two loci. Because of the similar MLGs and because a large number of neonates were collected in the same location on the same day (especially in the O.H. Ivie reservoir), there was the possibility that sibling groups were represented in the data set. As the presence of family groups is known to inflate estimates of population subdivision among *a priori* defined population units (Allendorf and Phelps 1981), I analyzed the data two ways. First, I used the full data set of 16 loci and 109 individuals. Second, I created a reduced data set that attempted to reduce potential sibling structure effects. COLONY (Wang et al. 2012) was used to identify potential full siblings. For the initial analysis, all neonate snakes were included. Neonates were identified based on total length for this life stage and collection date (classification of age group was based on snout-vent-length (SVL): adult males >380 mm SVL, adult females >420 mm SVL, juvenile females <420 mm SVL, neonates <250 mm SVL; Greene et al 1999). Male and female mating system was set to polygamy without inbreeding. The full-likelihood analysis method, the length of run was set to long, high likelihood precision and a weak sib-ship prior was used. False allele rate was held constant at 0.01 and four allele drop our rates were explored (0.1, 0.01, 0.05, and 0.001). Sibling groups were consistently identified at each technical

error rate with a 95% or greater probability (Table 4.4. The same process was repeated for juvenile and adult snakes but no siblings were identified above an 80% probability. All subsequent analyses were repeated for both the entire data set (N=109) and the reduced data set after the siblings had been removed (N=92).

Population Structure, Connectivity, and Isolation by Distance

As an initial assessment of potential population structure and genetic diversity, samples were grouped into 3 regions: the Upper Colorado River (UCR), Lower Colorado River (LCR), and O.H. Ivie Reservoir (OHIR). Samples from all three years were grouped into these three *a priori* delimited groups due to small sample sizes within each given sampling location and/or year. An overall test of genetic differentiation (and estimation of F_{ST}) along with pairwise tests of genetic differentiation (and pairwise estimates of F_{ST}) were conducted using the 16 loci among the three regions in FSTAT 2.39 (Goudet 2001). These tests rely on *a priori* delimitations of populations, thus we also conducted 3 individual-based clustering analyses. The Principal Coordinate Analysis (PCoA) was implemented in GENALEX 6.502 (Peakall and Smouse 2012). I used the model based Bayesian clustering implemented in STRUCTURE 2.3.4 (Pritchard et al. 2000), which partitions individuals based on HWE and linkage equilibrium. The input parameters for STRUCTURE were correlated allele frequencies, and the admixture model. STRUCTURE was run with 5,000,000 iterations with a burn-in of 500,000 iterations for *K* (i.e., the number of possible clusters) values 1 to 10 with 10 replications of each possible *K* value. 3) The spatial and non-spatial Bayesian clustering program implemented in the program BAPS 6.0 (Corander et al. 2008). Ten

iterations of each K from 2 to 10 were repeated. Clustering was performed at the level of the individual. In order to compare the results of this population structure analysis to the patterns found by Rodriguez et al (2012), a reduced dataset of the 3 cross amplified loci was used following the procedures described above for the 109 individuals collected in this survey.

Patterns of isolation by distance were examined by testing the correlation between genetic distance and geographic distance. The genetic distance matrix was created in genepop 1.0.5 for pairwise comparisons between individuals analogous to F_{ST} /(1- F_{ST}) in R (Rousett 2008). A geographic Euclidean distance matrix was generated in adegent 2.1.1. (Jambart 2008). The mantel test for isolation by distance for pairwise comparisons between individuals was implemented with 1000 permutations and visualized in adegenet 2.1.1 (Jambart 2008).

Effective Population Size and Detection of Bottlenecks

The population effective size (N_e) of each of the three regions was estimated using the biased-corrected linkage disequilibrium estimator of Waples (2006) as implemented in the software NEestimator (Waples and Do 2008). The primary assumption behind using the linkage disequilibrium method (Waples 2006) is that genetic drift is the only cause of the linkage disequilibrium in the sample. Thus, the method assumes a closed population (i.e., no migration; Waples and England 2011). If in the population sample there are a few migrants that originated from highly diverged populations, LDN*e* estimates of N_e may sometimes be decreased due to the linkage disequilibrium generated by admixed individuals. N_e estimates were performed for the

three groups identified in both STRUCTURE and BAPS. Thus the three estimates of local population effective size of effective population size were for the UCR, LCR and OHIR. The program COLONY (Jones and Wang 2010) was also used to estimate N_e. This program estimates the effective population based on the frequency of full-siblings and half siblings present in the sample. For this analysis neonate and juvenile *N. h. paucimaculata* were grouped as offspring genotypes. Neonate and juvenile designations were based on previous authors' size delineations (snout-vent-length (SVL): adult males >380 mm SVL, adult females >420 mm SVL, juvenile females <420 mm SVL, neonates <250 mm SVL; Greene et al 1999). Adult male and females were included as potential reproductive adults. The program parameters were set to the full-likelihood method, long run time, male and female polygamy, and no inbreeding. Error rates were varied as described in the sibling identification methods but did not alter the effective size estimates between runs. One potential drawback for this method was that it was possible for juveniles captured in 2013 and 2014 to be the parents of neonates captured in 2015. As it is possible for parent-offspring relationships to be incorrectly identified as sibling pairs and thus underestimate the number the effective population size, it was first verified that no adult-offspring pairs were present within the dataset between years with a 95% probability.

The program BOTTLENECK 1.2.02 (Piry et al. 1999) was used to test for the presence of recent bottlenecks among the three subpopulations identified by BAPS and STRUCTURE. Samples from the uppermost Colorado River sites were grouped due to small sample sizes and proximity of collection site. The Wilcoxon signed rank test was

used to test for an excess of gene diversity (i.e., heterozygosity under HWE) relative to the heterozygosity expected based on the observed allelic diversity under mutation-drift equilibrium. This test of a bottleneck is based on the concept that when a population undergoes a large reduction in effective population size (N_e) the decrease in number of alleles will occur at a faster rate than the gene diversity of the population. Bottlenecks were tested for with three mutation models, the infinite alleles model (IAM), step-wise mutation model (SSM) and two-phase mutation model (TPM) at 10,000 coalescent simulations. The TPM model was implemented at four different multistep change proportions (30, 10, 5, and 1%) and with the default variance of 30.

Results

Genetic Diversity and Equilibrium Tests

Sixteen microsatellite loci were amplified from all 109 *N. h. paucimaculata* sampled from 2013-2015. The mean number of alleles per locus was 3.625 for the UCR, 3.625 for the OHIR, and 4.036 for the LCR (Table 2.4). Three loci in the OHIR and LCR populations showed significant deviations from HWE, while four loci in the LCR showed significant deviations from HWE. The multilocus F_{IS} values were lowest for the OHIR -0.023 and highest for the upper Colorado River (0.220). The LCR population had a multilocus F_{IS} value of 0.110. There are 120 pairwise comparisons for 16 loci. At a nominal alpha level of 0.05, one would expect 6 of the 120 comparisons to exhibit linkage disequilibrium by chance alone. The UCR, 25 of the pairwise comparisons were significant and 18 were significant in the OHIR. The LCR, with only 8, had the lowest number of significant pairwise comparisons. There was no significant differences in

allelic richness between the three populations (p=0.929). Gene diversity (H_s) did not differ significantly among the three groups (p=0.7334). Average F_{IS} was also not significantly different among the three populations (p=0.331). The LCR had the most number of private alleles (8) followed by the UCR with 4 and the OHIR with 2.

The genetic diversity measures of the five loci used by Rodrigues et al. (2012), here after referred to as the Rodriguez loci, were also examined for comparative purposes. For the 109 Concho water snakes captured in this study, the average number of alleles from the Rodriguez loci was 3.4 for the UCR, 2.8 for OHIR, and 3.0 from the LCR (Table 3.4). Locus Ts3 and Ebou1 were found to be monomorphic within the samples collected for this study across all three sampling sites, while locus Nsu4 was monomorphic in the Upper and Lower Colorado River. Locus 3Ts showed significant deviations from HWE in the upper Colorado River, while Nsu3 had a significant deviation in the lower Colorado River. The highest number of private alleles was in the LCR (AP=3), and similarly low in both OHIR (AP=1) and the UCR (AP=2). Locus Ts3 and locus Ebou1 were previously found to be variable in this species (Table 3.4) (Rodriguez et al 2012). Thus, while the average number of alleles was similar between the loci from this study and the previously used loci, this pattern was driven by the higher number of alleles in Nsu3 and 3Ts.

Sibling Identification

Nine groups of full siblings were identified with a high probability (≥ 95%), ranging from pairs to a family group of 8 individuals (Table 4.4). All sibling groups consisted of neonate snakes captured during the fall sampling period in early September.

Most of the siblings were encountered at the O.H. Ivie reservoir location near the Concho recreation area. No half siblings were identified among the neonates. No full- or half-siblings were identified within either the juvenile or adult age classes. As full sibling groups of individuals could artificially inflate estimates of population structure, a single representative individual from each full sibling group was selected for the reduced data set, resulting in 92 individuals. All population structure analyses were performed on both the full data set (N=109) and the reduced dataset (N=92) to determine if the presence of siblings among the samples inflated population structure

Population Structure and Isolation by Distance

The 16 variable loci (13 new, 3 from Rodriguez et al. (2012)) were used in all estimates of population structure and isolation by distance. There was significant pairwise structure identified among the three populations (Table 5.5). The greatest genetic similarity based on F_{ST} values was between the upper and lower Colorado River populations (F_{ST}=0.062, p= 0.001) (Table 5.4). The OHIR harbored the most divergent population compared to the UCR (F_{ST}=0.125, p=0.001) or the LCR (F_{ST}=0.141, p=0.001).

Table 2.4. Average microsatellite diversity for the 109 Concho water snake (*N. h. paucimaculata* **)** at 16 loci (13 new, 3 variable loci from Rodriguez et al. (2012))the upper and lower Colorado River and from O.H. Ivie Reservoir.

Population	N^a	$A_N{}^b$	$P_A{}^c$	$A_R{}^d$	$H_O{}^c$	$H_S{}^d$	F_{IS}
Upper Colorado River	*22*	*3.625*	*4*	*3.625*	*0.515*	*0.517*	*0.220*
O.H. Ivie Reservoir	57	3.625	2	3.26	0.485	0.48	-0.023
Lower Colorado River	30	4.036	8	3.89	0.458	0.502	0.110

[a] Number of individuals genotyped

[b] The number of alleles per locus

[c] Number of private alleles

[d] Allelic richness

[e] Observed heterozygosity

[f] Gene diversity

Table 3.4. Average microsatellite diversity for the 109 Concho water snake (*N. h. paucimaculata*) at 5 loci used by Rodriguez et al. (2012).

Population	N[a]	A_N[b]	A_R[c]	H_O[d]	H_S[e]	F_{IS}
Upper Colorado River	*22*	*3.4*	*3.4*	*0.282*	*0.3106*	*0.093*
O.H. Ivie Reservoir	57	2.7202	2.8	0.0303	0.2966	-0.021
Lower Colorado River	30	3	2.927	0.25	0.2926	0.147
Rodriguez et al. 2012	34	4.2	NA	0.34	0.43	NA

[a] Number of individuals genotyped

[b] The number of alleles per locus

[c] Allelic richness

[d] Observed heterozygosity

[e] Gene diversity

Table 4.4. Neonate sibling groups identified in COLONY at 95% or above probability at four technical error rates (0.1, 0.01, 0.05, and 0.001). Individuals included in the reduced dataset are indicated by an X.

Individual ID	Family Group ID	Reach of the River	Collection Site	Included in the Reduced Dataset
NePa66	1	Lower Colorado River	Hwy 283	X
NePa68	1		Hwy 283	
NePa100	2	O.H. Ivie Reservoir	Concho Rec Area	X
NePa106	2		Concho Rec Area	
NePa105	3		Concho Rec Area	X
NePa110	3		Concho Rec Area	
NePa74	3		Concho Rec Area	
NePa79	3		Concho Rec Area	
NePa82	3		Concho Rec Area	
NePa85	3		Concho Rec Area	
NePa93	3		Concho Rec Area	
NePa98	3		Concho Rec Area	
NePa107	4		Concho Rec Area	X
NePa73	4		Concho Rec Area	
NePa91	4		Concho Rec Area	
NePa94	4		Concho Rec Area	
NePa112	5		Concho Rec Area	
NePa75	5		Concho Rec Area	
NePa81	6		Concho Rec Area	X
NePa88	6		Concho Rec Area	
NePa57	7	Upper Colorado River	12 Mile Bridge	X
NePa58	7		12 Mile Bridge	
NePa60	8		12 Mile Bridge	X
NePa64	8		12 Mile Bridge	
NePa71	9		Hwy 83	X
NePa83	9		Hwy 83	

The PCoA supported the pairwise differentiation tests, with the three groups largely corresponding to the geographic clustering of samples in the multivariate space of axes 1 and 3 of the PCoA figure (Figure 3.4). The greatest principle components axis explained 38.77% (1 vs 3) of the variation separating the OHIR population from the other reaches on the Colorado River.

The results of the Bayesian clustering analysis using 16 loci implemented in STRUCTURE were largely concordant with both the results of the pairwise F_{ST} tests and the PCoA as there is visible structure evident between the three groups for a K of 3 (Figure 4.4a). The OHIR population is visibly divergent at K=2 from both the UCR and LCR regions. At *K*=4, the population substructure associated with the collection sites is apparent. Also, note that in both the PCoA and the STRUCTURE results, some individuals from the UCR cluster with the LCR. The Bayesian clustering program implemented in BAPS using individual clustering indicated that the most likely number of genetic clusters was 4 in both the spatially explicit and the non-spatially explicit models (Figure 4.4b). Again some individuals from the most upstream sampling locations clustered with the lower Colorado River samples. The clusters from the BAPS analysis largely corresponded to individuals collected from the same riffle, indicating local levels of population structure strongly associated with the riffle sites where the snakes were captured (Figure 4.4). The exception was cluster 3 (Figure 4.4.b) which included samples from the two most upstream collection sites (Hwy 277 and Hwy83-blue) and the most downstream collection site (Hwy 283-blue). The analysis was then repeated using the 16 loci but with sibling groups removed from the data set.

Table 5.4. Pairwise measures of F_{ST} based on sixteen microsatellite loci for the complete data set (N= 109) below the diagonal. Significance values given above the diagonal based on Weir and Cockerham (1984) theta estimate set for 999 permutations.

	UCR	OHIR	LCR
UCP	-	**0.001**	**0.001**
OHIR	0.125	-	**0.001**
LCR	0.062	0.141	-

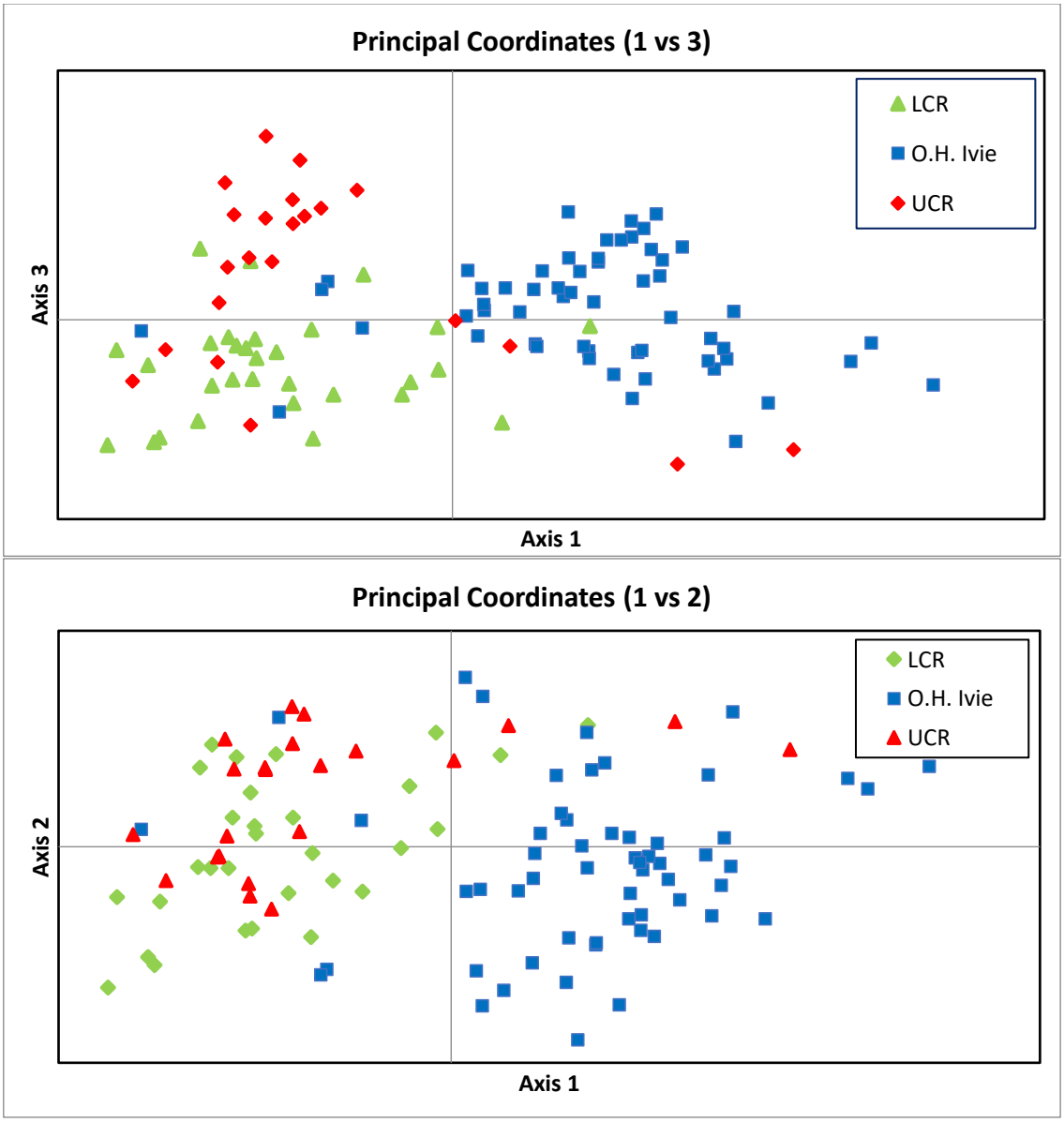


Figure 3.4. PCoA shown for axis 1 vs. 3 (35.77 % of the total variance) and Axis 1 vs. 2 (27.33% of the total variance) for 109 individuals based on information from 16 loci. Three delimited clusters are noticeable in the PCoA: lower Colorado River (LCR, (green), upper Colorado River (UCR, red) and the O.H. Ivie reservoir (O.H. Ivie, red). Axis 1 separates the OHIR population from the other reaches of the Colorado River. Axis 3 largely delimits the UCR and LCR populations.

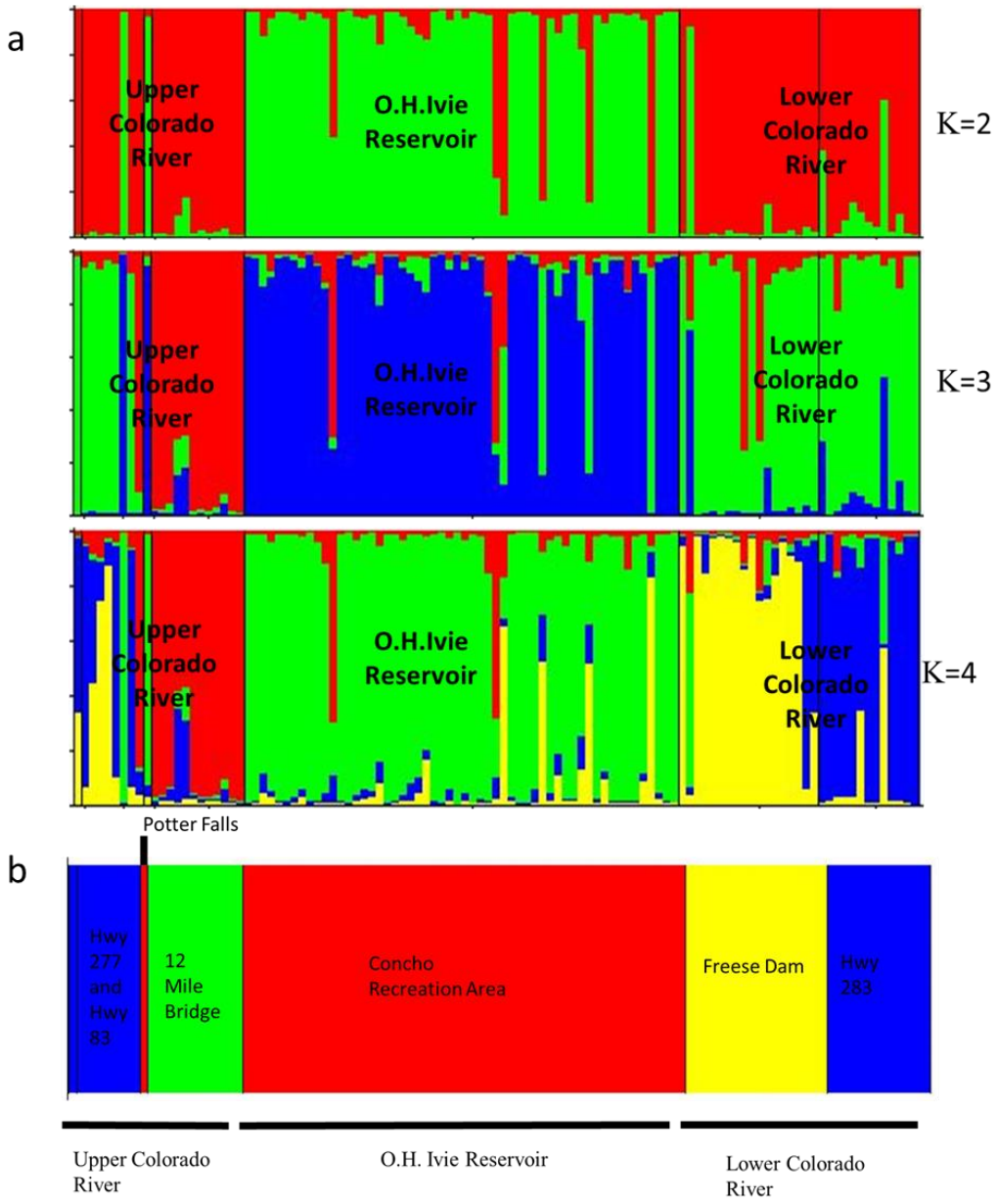


Figure 4.4. a. Results from the Bayesian clustering analysis in STRUCTURE for 109 *N. h. paucimaculata* individuals using 16 microsatellite loci. Individuals are arranged from most upstream to most downstream collection site. Each color represents a genetic cluster and each bar represents an individual's proportional association with that cluster. **b**. Results of the BAPS Bayesian clustering analysis mixture partitioning. The program identified 4 as the most likely number of clusters with a probability of 0.97.

When sibling groups were removed from the dataset, the patterns of population subdivision were virtually identical (Figure 5.4 and Figure 6.4). Populations from the upper and lower Colorado again exhibited the lowest, but still significant, population subdivision (F_{ST}=0.055, p=0.001) (Table 6.4). F_{ST} values were highest between the Upper Colorado River and O.H. Ivie reservoir (F_{ST}=0.136, p=0.001) and the lower Colorado River and O.H. Ivie (F_{ST}=0.146, p=0.001). The results from the PCoA, STRUCTURE and BAPS analysis were not altered substantially by the removal of the sibling groups (Figures 5.4 and 6.4) with the exception of the upper Colorado River cluster which was more uniformly admixed with the lower Colorado clusters, a pattern that was also apparent at K=4.

When the population structure analysis was conducted using the Rodriguez loci for the full data set of 109 Concho water snake individuals , pairwise measures of F_{ST} between the lower Colorado River and O.H. Ivie were lower, but still significant (F_{ST}=0.088, p=0.001) (Table 7.4). Pairwise F_{ST} estimates between the Upper Colorado River and O.H. Ivie (F_{ST}=0.124, p=0.001) and the upper and lower Colorado River (F_{ST}=0.091, p=0.001) were slightly altered compared to the full data set of 16 markers. A reduction in the detection of population substructure with the reduced dataset was observed with the results of the PCoA and STRUCTURE analysis (Figure 7.4 and Figure 8.4). The three reaches of the river were not clearly delineated in the PCoA (Figure 7.4). The program STRUCTURE was unable to differentiate among populations using only the Rodriguez loci and yielded an erroneous result. The analysis from BAPS however identified five clusters, again delineating genetic clusters associated with

survey sites. At two locations, Hwy 277 and Potter Falls, only a single individual was encountered at each site. The individuals from Hwy 277 and Potter Falls were variable in the genetic clusters that they were associated with between analyses.

The results of the mantel test showed a weak but significant pattern of isolation by distance (r=0.28, p=0.001) (Figure 9.9). The overall pattern of isolation by distance was reduced by individuals from the Hwy 277 and Hwy 83 on the Upper Colorado River which had higher genetic similarity to individuals collected from Hwy 283, the most downstream sampling location, despite their greater geographic distance. This pattern was also observed in the STRUCTURE and BAPS analysis.

Effective Population Size and Detection of Bottlenecks

The local effective population sizes calculated using the LDN*e* method was estimated to be the highest for the survey sites in OHIR, followed by the LCR. (Table 8.4). Sites located on the upper Colorado River had the lowest local effective size of 8.6. Estimates of local effective sizes carried out in COLONY were higher for all populations (Table 8.4), but fell within the confidence intervals for local effective sizes estimated in LDN*e* (Table 9.4).

An excess of heterozygotes indicative of recent bottlenecks was detected using the Wilcoxon test in the Uppermost Colorado River sites (Hwy 277, Hwy 83, and Potter Falls) and in the O.H. Ivie reservoir for both the IAM and TPM models at all four multistep change proportions (Table 10.4). Bottlenecks were not detected at any of the sites under the SSM model.

Table 6.4. Pairwise measures of F_{ST} based on sixteen microsatellite loci for reduced data set (N=92, no siblings) below the diagonal. Significance values given above the diagonal based on Weir and Cockerham (1984) theta estimate set for 999 permutations.

	UCR	OHIR	LCR
UCP	-	**0.001**	**0.001**
OHIR	0.124	-	**0.001**
LCR	0.053	0.128	-

Table 7.4. Pairwise measures of F_{ST} based on three variable loci (Rodriguez et al. 2012) for the complete data set (N=109) below the diagonal. Significance values given above the diagonal based on Weir and Cockerham (1984) theta estimate set for 999 permutations.

	UCR	OHIR	LCR
UCR	-	**0.001**	**0.001**
OHIR	0.124	-	**0.001**
LCR	0.091	0.088	-

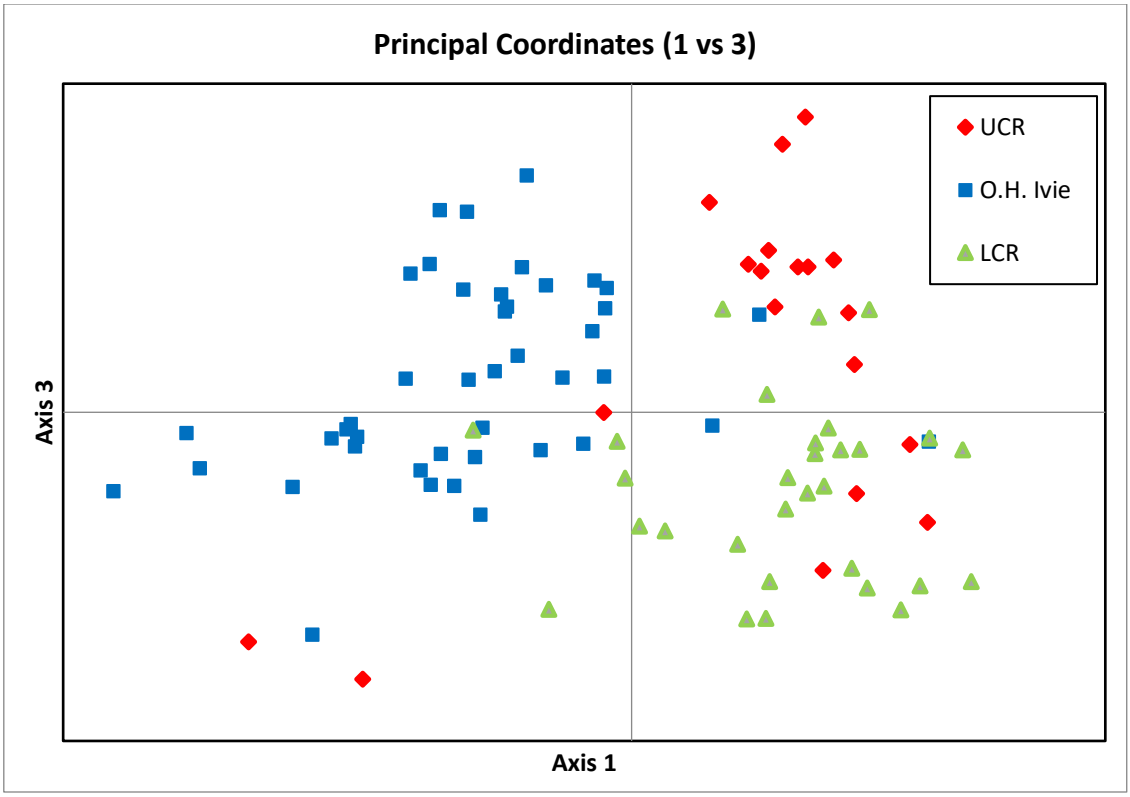


Figure. 5.4. PCoA shown for axis 1 vs. 3 (35.77 % of the total variance) for the reduced data set of 92 *N. h .paucimaculata* individuals using 16 microsatellite loci. Three clusters associated with the three sections of the river are delimited. Individuals from the upper Colorado River fall outside of the main red cluster.

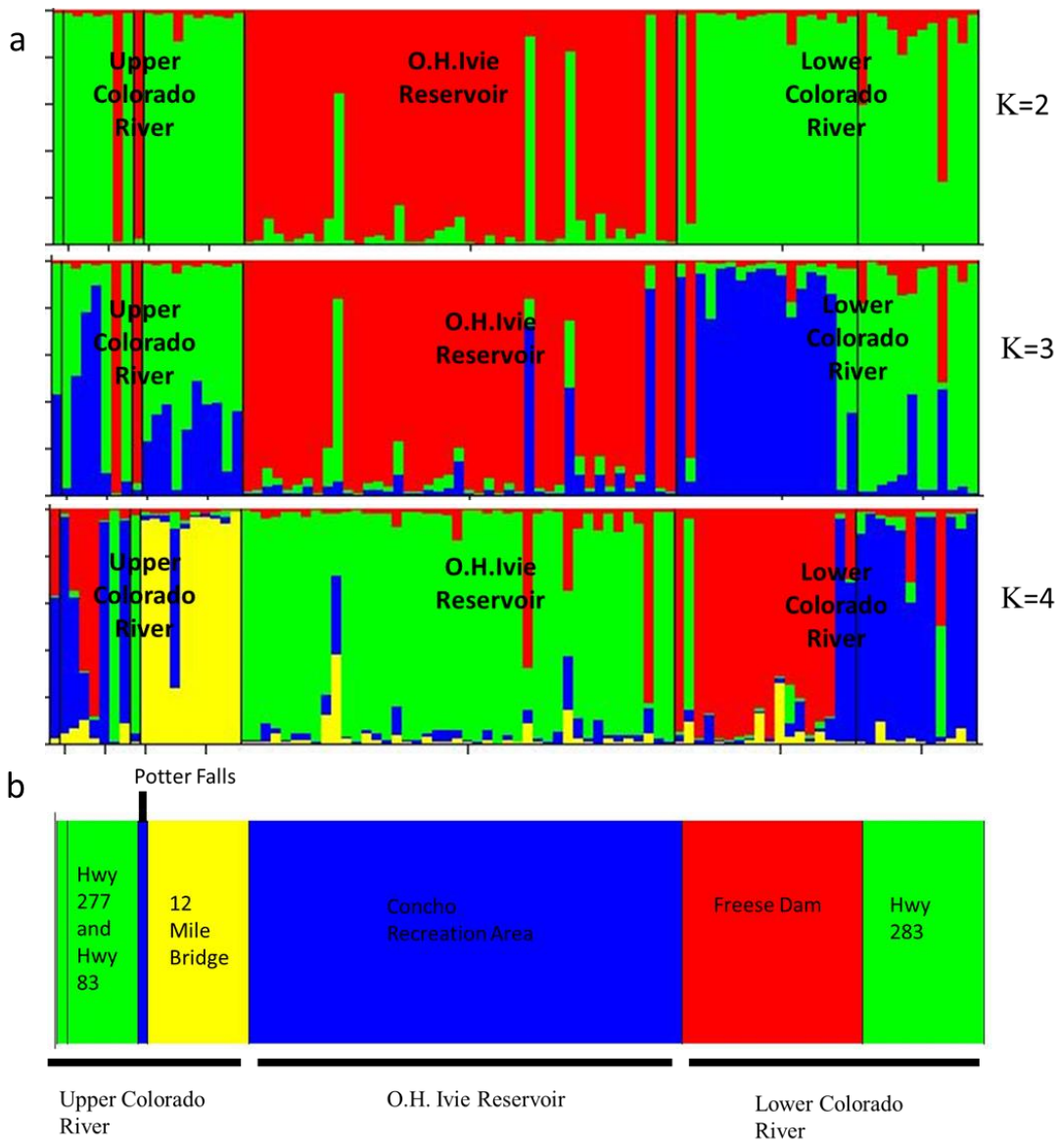


Figure 6.4. a. A plot from the Bayesian clustering analysis in STRUCTURE for the reduced data set with siblings removed (N=92) *N. h. paucimaculata* individuals using 16 microsatellite loci. Individuals are arranged from most upstream to most downstream collection site. Each color represents a genetic cluster and each bar represents an individual's proportional association with that cluster. **b.** Results of the BAPS Bayesian clustering analysis mixture partitioning. The program identified 4 as the optimal number of partitions with a probability of 0.96.

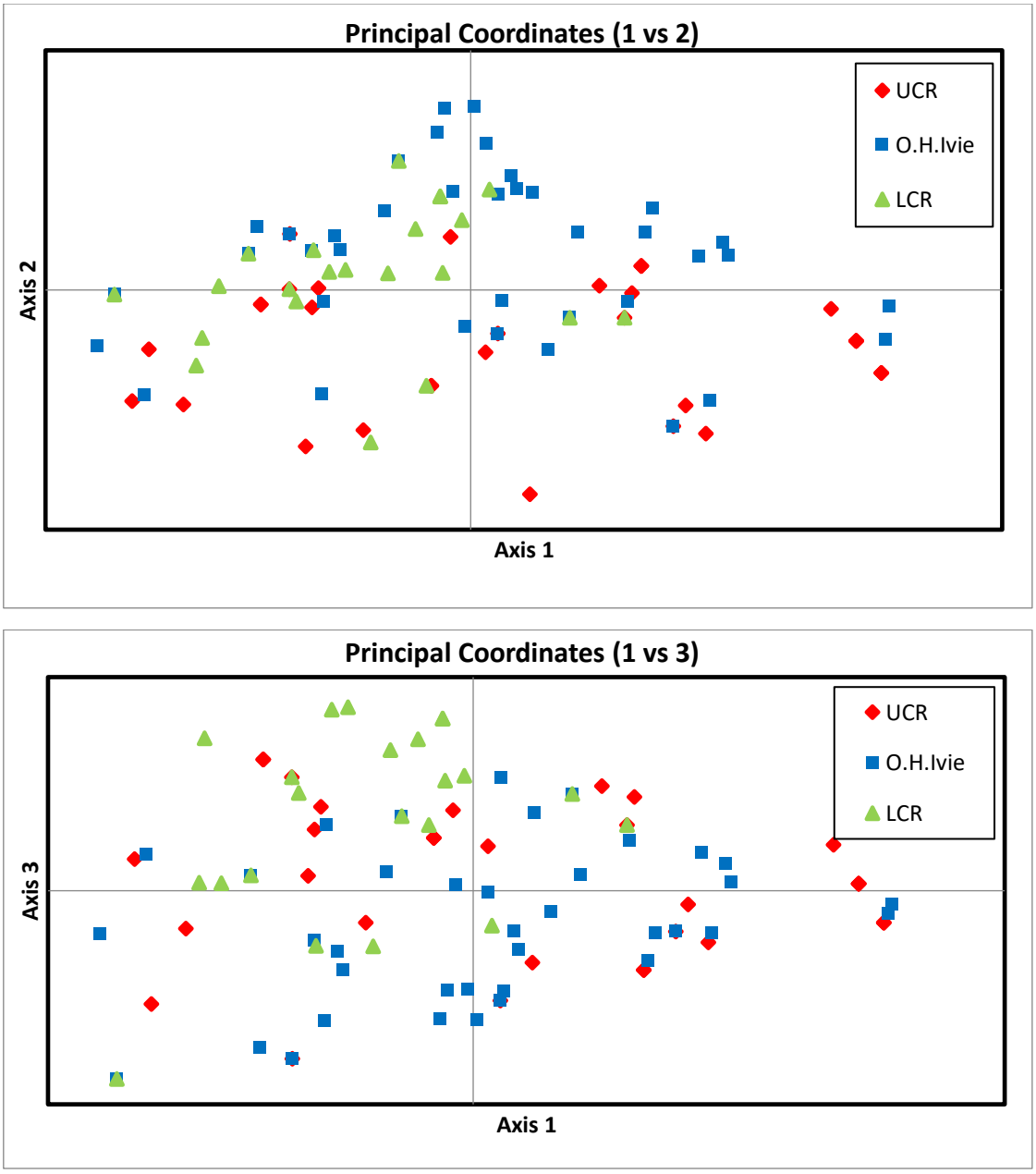


Figure 7.4. PCoA shown for axis 1 vs. 2 (37.33 % of the total variance) and 1 vs 3 (55.29% of the total variance) for 109 individuals based on information from the Rodriguez loci. Note that distinct regional clusters are no longer detected for either axis comparison.

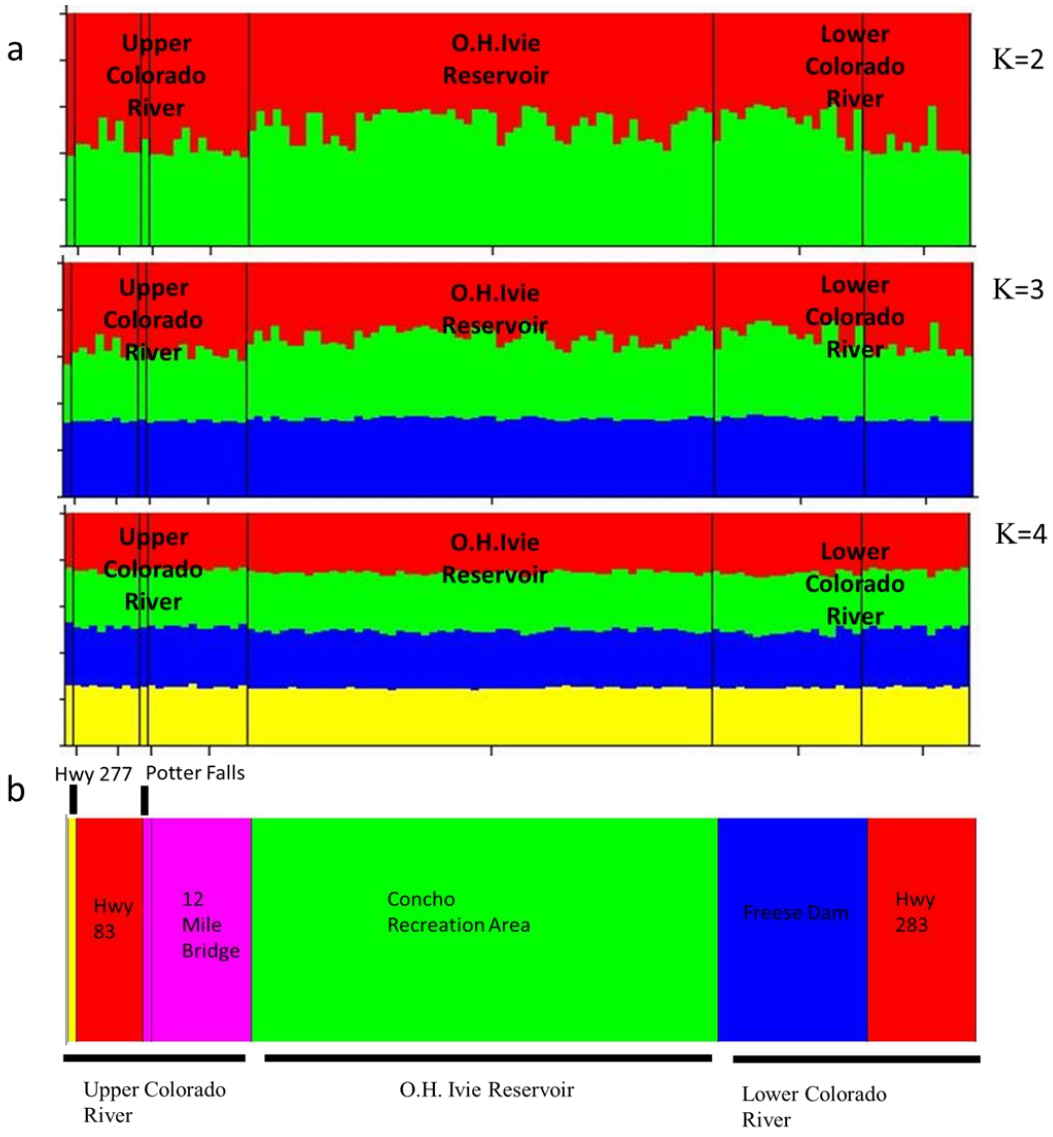


Figure 8.4. a. Results from the Bayesian clustering analysis in STRUCTURE for 109 *N. h. paucimaculata* individuals using the Rodriguez loci. Individuals are arranged from most upstream to most downstream collection site. With only the 3 cross amplified loci, the program was unable to detect the patterns of substructure observed with the 16 combined loci. **b**. Results of the BAPS Bayesian clustering analysis mixture partitioning. The program identified 5 as the optimal number of partitions with a probability of 0.96.

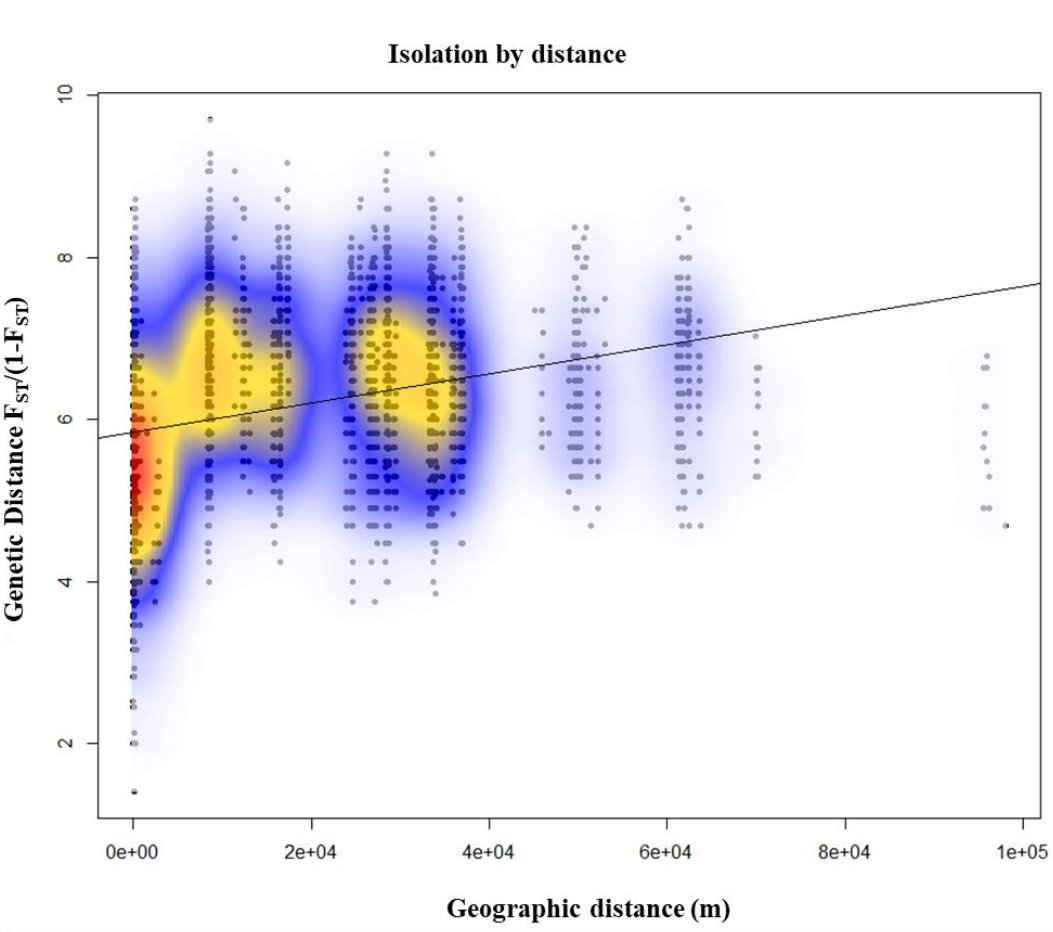


Figure 9.4. Correlation between geographic distance (m) and pairwise comparisons between individuals analogous to F_{ST} /(1- F_{ST}) (Rousett 2008) on the Colorado River and O.H. Ivie Reservoir (Mantel Test, r=0.286, p=0.001). Analysis based on 16 microsatellite loci from 109 *N.h.paucimaculata* individuals. Clusters of individuals indicative of the underlying population structure between riffle sampling locations.

Table 8.4 Effective population size estimates using LDN*e* method for the full data set (N=109) and the 16 combined microsatellite loci with groups of samples clustered by population clusters identified in PCoA and STRUCTURE. Lowest allele frequency cutoff was 0.03 for LCR and O.H. Ivie, and 0.02 for UCR based on the guidelines of Waples and Do (2010).

	N	r^2	N_e	Confidence Intervals
Upper Colorado River	22	0.16897	8.6	5.9-12.4
O.H. Ivie Reservoir	56	0.040	13.1	10.2-16.8
Lower Colorado River	31	0.058	12.2	8.9-16.8

Table 9.4 Effective population size estimates from COLONY. The program parameters were set to the full-likelihood method, long run time, male and female polygamy, no inbreeding for the full data set (N=109) and 16 loci. Error rates were varied as described in the sibling identification methods but did not alter the effective size estimates between runs.

	N	*Alpha*	N_e	Confidence Intervals
Upper Colorado River	22	0.00	18	10-36
O.H. Ivie Reservoir	56	0.00	17	10-36
Lower Colorado River	31	0.00	19	11-38

Table 10.4. Detection of bottlenecks in survey sites estimated by an excess of heterozygotes. Bold values indicate statistically significant excess of heterozygotes ($\alpha < 0.05$).

Survey Site	**N**[a]	**IAM**[b]	**TPM**[c]				**SSM**[d]
			30	10	5	1	
Upper Colorado River	22	**0.001**	0.04	0.04	0.04	0.04	0.51
O.H. Ivie Reservoir	56	**0.001**	**0.01**	**0.003**	**0.003**	**0.003**	0.52
Lower Colorado River	31	**0.01**	0.36	0.38	0.56	0.41	0.09

[a] Number of individuals

[b] Infinite alleles model

[c] Two-phased mutation model assessed at four multistep change proportions

[d] Step-wise mutation model

Discussion

Genetic Diversity

The Concho water snake is a habitat specialist that occupies at most approximately 450 km of river and reservoir habitat in west-central Texas. Microsatellite diversity was moderate and not significantly different between the upper and lower Colorado and O.H. Ivie reservoir. Of the five cross amplified loci included in both this study and that of Rodriguez et al. (2012), two loci were monomorphic and one was monomorphic in all but a single individual. Levels of genetic diversity detected in this study are similar or lower than other snake speciesthat have undergone recent bottle necks or isolation due to habitat loss or degradation such as the Copperbelly water snake (*N. erythrogaster neglecta*) (Marshall Jr. et al. 2009), Western Massasauga (*Sistrurus catenatus*) (Gibbs and Chiucchi 2012, McCluskey and Bender 2015), Smooth snakes (*Coronella austriaca*) (Pernetta et al. 2001) and eastern Fox snakes (*Pantherophis gloydi*) (Row et al. 2010).

Population Structure and Isolation by Distance

I detected population differentiation and significantly differentiated F_{ST} values among the Upper and Lower Colorado River and O.H. Ivie Reservoir. The O.H. Ivie Reservoir population is the most divergent population. Overall, the 3 *a priori* delimited regions are supported by genetic clusters recovered by the programs that ignore geographic information. The UCR showed some admixture, containing some individuals similar to the LCR in addition to individuals that form a unique cluster. The O.H. Ivie reservoir population forms its own unique cluster in each analysis. Clusters of

individuals identified by both STRUCTURE and BAPS are largely associated with riffle systems and hence survey sites along the Colorado River indicating there is underlying structure between populations along the river and weak patterns of isolation be distance. The individual captured at Potter Falls was most variable in its assignment, grouping variously with the O.H. Ivie Reservoir and 12 Mile Bridge clusters potentially due to recent dispersal or the lack of statistical power associated with identifying the placement of a single individual. The collection of additional samples from this riffle may prove useful in determining the genetic placement of snakes from this riffle location. Overall, our results support the characterization of *N. h. paucimaculata* as a species with high site fidelity. The high divergence associated with the reservoir indicates limited migration between this population and the upper and lower Colorado River populations.

Two unexpected patterns are evident in our analysis of population structure. Individuals from the uppermost sites on the Colorado River (Hwy 277 and Hwy 83) consistently clustered with individuals from the lower Colorado River from riffles downstream of Hwy 283 but not with individuals from any of the subpopulations in between. There is a distance of approximately 108 river kilometers between the Hwy 277 river crossing and the riffles downstream of Hwy 283 with O.H. Ivie reservoir between, thus long-distance migration between the two locations is unlikely. This pattern may be indicative of past successful translocation activities. The Colorado River Municipal Water District, which manages the regions' reservoirs, committed to carrying out translocations between the upper and lower Colorado River as part of the proposed species delisting (USFWS 2011). Information regarding the timing of the translocations,

the locations and number of individual snakes involved was undocumented but the patterns of population structure detected in this study suggest that the translocated individuals or their resultant offspring are occupying the upper reaches of the Colorado River.

The second unusual pattern I detected was the degree to which the population within O.H. Ivie Reservoir differentiated from the populations on the upper and lower Colorado River. Rodriguez et al. (2012) captured 34 snakes spanning a large region of the upper Colorado River and 30 snakes from the Concho River. They found an F_{ST} of 0.27 (based on 5 microsatellite loci) between these 2 regions and they found 2 mtDNA haplotypes, one largely associated with the Colorado River and the other more associated with the Concho River. The F_{ST} values were lower for both the 16 combined loci (UCR to O.H.Ivie, 0.125 and 0.141) and the five cross amplified loci (0.136, 0.146) for the pairwise comparisons between the upper Colorado and O.H. Ivie reservoir and the lower Colorado River and O.H. Ivie reservoir but were approximately three fold greater than F_{ST} values between the upper and lower Colorado River populations (0.055). Given that the O.H. Ivie reservoir population is located slightly less than 1 mile downstream of the confluence between the Colorado and the Concho River, it is possible that the population subdivision between the UPC-LCR to the O.H. Ivie reservoir population is driven by a descendent Concho River population. However despite extensive sampling, I was unable to directly test this hypothesis as I did not encounter Concho water snakes on the Concho River. Additional mitochondrial sequencing of the

samples collected during this study may help to determine the origin of the OHIR population.

Eight groups of full siblings were identified within the data set however their removal did not significantly affect the results of the population substructure analysis. This was likely due to their small number in proportion to the complete data set. However, as fall surveys during the year with the highest precipitation yielded the greatest catch per unit effort and sibling groups were only detected in samples of neonates captured in the fall, future monitors should be conscious of the effect that large numbers of neonates and potential siblings may have on estimates of population structure from fall-based survey efforts. Furthermore, the results of future structure analysis based on fall sampling seasons alone may not be an accurate reflection of the adult population structure due to low neonate survivorship and the potential to overestimate structure when sibling groups are sampled (Allendorf and Phelps 1981; Whiting et al. 2008).

The reduced data matrix of the Rodriguez loci yielded similar pairwise F_{ST} values between the upper and lower Colorado River and between the upper Colorado River and O.H. Ivie Reservoir. However the F_{ST} values between the Lower Colorado River and O.H. Ivie Reservoir for the reduced data set under estimated subdivision between the O.H. Ivie reservoir and the lower Colorado. With just the loci used by Rodriguez et al. (2012) the program STRUCTURE failed to identify population differentiation among the sampling sites or three regions of the river based on the reduced data matrix, while BAPS identified 6 as the most likely number of genetic

clusters. Thus the full dataset of 16 loci is required to achieve greater consistency between clustering methods to assess levels of population structure and to identify fine-scale patterns of population subdivision among riffles.

Effective Population Size and Bottlenecks

Bottlenecks were detected in the groups of individuals from the three uppermost sites on the Colorado River and from the O.H. Ivie reservoir under both the IAM and TPM model at different variances. The upper Colorado River has very likely been most effected by drought. Data collected by the USGS monitoring gauges estimate that between 2010 and 2014 there were between 160 and 229 days each year were stream flow was absent from the Colorado River between Robert Lee and Ballinger, TX. Hence the detection of bottlenecks at these sites are consistent with the low precipitation in these locations. The subpopulation of Concho water snakes from the O.H. Ivie reservoir yielded the largest number of individuals from the post-delisting monitoring surveys, the majority of whom were neonates. The detection of a recent bottlenecks from populations in the O.H. Ivie reservoir could be linked to the recent inundation and subsequent colonization of the reservoir. However, previous authors have noted that Concho water snakes occurred at low densities within the reservoir, making them vulnerable to extirpation and were observed to move between locations frequently to accommodate fluctuations in water level (Whiting et al. 2008). Changes in reservoir habitat availability were also responsible for some of the longest movements recorded in radio telemetry studies (Whiting et al. 2008). Information from additional

subpopulations within the reservoir will be required to determine how local population dynamics may be affecting the genetics of reservoir populations.

Overall, significant amounts of linkage disequilibrium was observed in the regions. Linkage disequilibrium can be generated by genetic drift (i.e., small N_e) and/or admixed populations (this assumes the microsatellites are not under a common selective pressure given the library was randomly generated). Admixture in the UCR is supported by the results of the clustering analyses (Figures 2.4 and 3.4). The effect of admixed samples on the LDN*e* method of estimating N_e was analyzed by Waples and England (2011). The LDN*e* method may overestimate local N_e if there is a migration rate greater than 0.1 or underestimate N_e if a there are a few migrants from a highly diverged population. I cannot tease these factors apart because sample sizes would be too small if one was to separate the UCR into each sample site. Estimates of N_e from both COLONY and LDN*e* were low compared to estimates from other species in small isolated populations (Chiucchi and Gibbs 2010; Jansen et al. 2007). Both estimates from *LDNe* and COLONY suggest that the local effective breeding populations are small and on the order of 10-20 individuals within each of *a priori* delimited sampling areas. Scott et al. (1989) estimated 1-3 adult females contributed to the yearly recruitment of Concho water snakes at each riffle site, an estimate consistent with the low effective size estimates from this study.

Conservation Implications and Future Monitoring Efforts

As genetic monitoring inherently requires repeated sampling efforts, the results of this initial monitoring effort are not meant to act as a single, conclusive population

size estimate or conclusion regarding the status of the Concho water snake as a delisted species. Rather, they are meant to lay the ground work for future genetic monitoring surveys and to establish an initial framework for research-based population management. The results of this initial population genetic survey indicate that populations of Concho water snakes are isolated between the UCR, LCR and OHIRs and exhibit additional local population substructure associated with riffle systems. Long periods of drought may have contributed to the bottlenecks detected. I was unable to directly test for the effects of O. H. Ivie reservoir in the absence of historical genetic data but the alterations in hydrology may be a major obstacle for future conservation efforts.

Previous conservation and management efforts in other systems have emphasized the importance of adaptive monitoring (Lindenmayer and Likens 2010; Lindenmayer et al. 2011). An adaptive monitoring program is one wherein the development of additional questions, experimental design and monitoring data collection are intrinsically determined by the results of previous efforts. Successful monitoring programs are required to adapt to new questions, and account for variation in conditions and information to improve future monitoring efforts. The first two years of the post-delisting monitoring efforts overlapped with a five year drought during which time no water was in the Colorado River upstream of the Hwy 277 crossing and downstream of Spence Reservoir. These conditions persisted in 2015 after the drought conditions ended downstream of Hwy 83 south of Ballinger, TX. Water snakes are highly dependent and specialized for life in aquatic systems. As such they are susceptible to prolonged periods of drought, which is exacerbated by the presence of reservoirs. Scott et al. (1989) and

Whiting et al. (2008) noted significant decreases in *N. h. paucimaculata* detectability during prolonged periods of drought as was also observed in this study; however, the biological responses of *N. h. paucimacualta* to drought are unknown. Research conducted by Winne et al. (2006) and Willson et al. (2006) suggest that migration and/or aestivation are common behavioral responses of aquatic snakes in response to drought. Aestivation during drought is known to be employed by another small-bodied highly aquatic snake, *Seminatrix pygea* (Winne et al. 2001) but is associated with high survival costs, especially to females, during prolonged droughts. Migration is employed by several species of *Nerodia* that occupy seasonally variable wetland habitats (Willson et al. 2006). Previous work on *N. h. paucimaculata* has also documented migration driven by habitat availability within reservoirs (Whiting et al 1997). However, prolonged periods of drought in river systems surrounded by arid, unsuitable habitat has been documented to cause significant decreases in year to year survival, slowed somatic growth, loss of large individuals from the populations and local extinction (Rose and Todd 2014; 2017). Currently, it is unknown if Concho water snakes are employing aestivation and/or, migration in response to the prolonged drought on the Colorado River. However, because there are no population size estimates prior to the delisting, it is not possible to determine if population sizes have decreased or if sampling was negatively impacted by extreme weather conditions. Additional future sampling seasons will be required to determine if the small sample sizes reflect snake activity in relation to weather conditions or if the snake populations do indeed have small census population sizes. Although not directly comparable, I note that historical studies (between years

1987-1996) examining the natural history of the Concho snake were able to obtain samples sizes upwards of 300 individuals in a given year (Whiting et al. 2008). Future research should address the behavioral responses and demographic impacts of drought on the Concho water snake to help better guide monitoring efforts in the future.

Conclusions

I determined that the Concho water snake exhibits population structure and isolation by distance along the Colorado River and within O.H. Ivie reservoir. The significant population structure between O.H. Ivie reservoir and the rest of the Colorado River indicates that there is little gene flow between this population and the upper and lower Colorado Rivers. As other authors have noted, Concho water snakes are able to persist within the reservoir but the detection of bottlenecks for this population suggests that it may have experienced recent reductions in size. Small effective population sizes and evidence of recent bottlenecks, coupled with low genetic diversity suggest that persistence of small isolated riffle populations in the upper Colorado River and the isolated populations within the reservoir may be greatly increased by translocation efforts from larger populations on the lower Colorado River. The presence of O.H. Ivie reservoir within the core range of the snake likely exacerbates existing patterns of population structure. The reduction in habitat quality and quantity caused by changes in flow regime and periods of drought are likely to contribute to reductions in Concho water snake population size.

CHAPTER V

SUMMARY

The research presented in my dissertation explores and elucidates the ecological and life history factors that can shape parasite population genetic structure at both the local and regional scales. The complexity of host-parasite systems is such that is it often difficult to disentangle ecological patterns and process without investigating both parasite and host in concert. In this dissertation I also address the population structure and connectivity of the Concho water snake (*N. h. paucimaculata*) with special interest in how the population genetics of this host can inform future conservation efforts on the Colorado and Concho Rivers and within O.H. Ivie Reservoir. By combining investigations of host-parasite population genetics, my research begins the processes of more accurately isolating the potential drivers of and barriers to host and parasite gene flow for R. aniarum and R. ancistrodontis and their snake definitive hosts.

Chapter II found that the population structure and effective population structure of *R. ancistrodontis* did not support the predictions of Nadler's hypothesis regarding the genetic consequences of host specificity. These results emphasize that additional life history characteristics such as life cycle complexity and aquatic transmission may override the influence of limited definitive host range in shaping parasite population structure and population effective size.

Chapter III demonstrated that *R. aniarum* population structure was determined by a combination of host dispersal and patterns of isolation by distance associated with sites

located along the distal branches of sites within a dendritic ecological network. Unusual patterns of population structure associated with parasites collected on the tributaries to the Colorado and Concho Rivers and immediately below O.H. Ivie Reservoir suggest that these sites are more strongly affected by local transmission dynamics than sites located on the main-stem of the rivers.

Chapter IV provided fine-scale patterns of population subdivision for the Concho water snake. The low population effective sizes and significant population subdivision between the upper Colorado River, lower Colorado River and within O.H. Ivie reservoir combined with small local population effective sizes suggest that the Concho water snake warrants additional monitoring and reassessment to ensure that the delisting of the snake was warranted.

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