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Cover Page Footnote

I wish to thank the USDA Forest Service San Bernardino, the California Department of Fish and Wildlife, the Associated Student Body, Inc., California State University, San Bernardino, the Biology Department of California State University, San Bernardino and the Water Resources Institute, California State University, San Bernardino for logistical support and funding. I would also like to thank my fellow lab members of the Metcalf lab; Liane Greaver, Jay and Pia Van Meter, and Joe Riley for assistance in the DNA extractions and PCR preparations for the microsatellite identification and characterization. I would also like to acknowledge the contributions of experience and funding support provided by my advisor, Dr. Tony Metcalf.

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Abstract
Geographic isolation and habitat fragmentation can cause a population to undergo independent evolutionary trajectories. Stream dwelling vertebrates such as the minnows belonging to the Cyprinidae family, one of the most genetically variable and geographically distributed groups, are excellent models for examining the genetic effects of population isolation and substructure. *Rhinichthys osculus*, a freshwater minnow, is a local species of concern that inhabits the Santa Ana and San Gabriel watershed systems within the Southern California region. 23 polymorphic microsatellite loci were characterized and identified for *Rhinichthys osculus* in conjunction with the Savannah River Ecology Lab. These microsatellite loci have been visualized in local lab settings. Currently over 150 samples of *Rhinichthys osculus* are being genotyped for each of the microsatellite loci to examine various population genetic parameters. The statistical analyses of the data will assist in potential conservation management.

Author Interview

Which professors (if any) have helped you in your research?
Dr. Tony Metcalf.

What are your research interests?
My research interests are population genetics, molecular ecology, and evolution.

What are your plans after earning your degree? What is your ultimate career goal?
After earning my degree I plan on pursuing a PhD in Ecology & evolution. I ultimately want to teach as a university professor while consulting for government agencies such as Fish & Wildlife and the U.S. Forest Service.

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I wish to thank the USDA Forest Service San Bernardino, the California Department of Fish and Wildlife, the Associated Student Body, Inc., California State University, San Bernardino, the Biology Department of California State University, San Bernardino and the Water Resources Institute, California State University, San Bernardino for logistical support and funding. I would also like to thank my fellow lab members of the Metcalf lab; Liane Greaver, Jay and Pia Van Meter, and Joe Riley for assistance in the DNA extractions and PCR preparations for the microsatellite identification and characterization. I would also like to acknowledge the contributions of experience and funding support provided by my advisor, Dr. Tony Metcalf.

KEYWORDS: *Rhinichthys osculus, Illumina, Microsatellite, PCR Primers, SSR, STR, Conservation*

Introduction

Stream dwelling vertebrates, in particular fish, and provide an excellent model for examining population substructure and genetic differentiation. Gene flow between tributaries and watersheds normally only occurs during times of flooding allowing for these populations to be geographically isolated from one another for extended periods. Each population may be independently influenced by various evolutionary forces due to their isolation causing for independent evolutionary lineages of particular genes to form. These genes become a part of the population's gene pool through mutations that randomly occur but then become unique to the population due to the extended period of isolation and habitat fragmentation.

Figure 1: Rhinichthys osculus, the Santa Ana Speckled Dace (University of California Agriculture and Natural Resources, 2012).

Rhinichthys osculus, the speckled dace (Figure 1), is considered to be one of the most ubiquitous freshwater fish in the Western United States and occupies a variety of environments (Hubbs, Miller, & Hubbs, 1974). In the Western United States, the speckled dace is the only native fish to be represented in all 7 drainage systems (Miller, 1958). Locally, the speckled dace is found in such creeks as Lytle Creek, Cajon Creek, City Creek and Mill Creek. *Rhinichthys osculu*s belongs to the *Cyprinidae* family, one of the most diverse families of freshwater fish. The species belonging to the *Cyprinidae* family inhabit a variety of environments including lakes, ponds, creeks, tributaries and even isolated springs across North America. Each of these environments contains different characteristics and histories. The Cyprinid family's ability to adapt to habitat changes in evidenced by their continued success in these ever changing environments, which may be in part, due to the increased genetic variability within the family (Moyle & Marchetti, 2006). Due to the highly variable characteristics of these habitats, the species of the *Cyprinidae* family have proven to be relevant models to address environmental and ecological changes from an evolutionary perspective (Scott & Crossman, 1973; Simons, Berendzen, & Mayden, 2003).

The Santa Ana Speckled Dace, the local variation of *Rhinichthys osculus,* is a small, cyprinid fish approximately 80mm in length and inhabits environments containing shallow cobble, gravel riffles and mixed sand habitats with overhanging riparian flora which provide a defensive measure to the fish from predators (Moyle & Marchetti, 2006); although they can inhabit environments that are fairly exposed, as well. The Santa Ana Speckled Dace once occupied the majority of the Santa Ana, San Gabriel and Los Angeles River systems but due to anthropogenic effects, their habitat has become highly fragmented. They were reported extirpated from the Los Angeles River system in the early 1990's which was most likely attributed to the urbanization of the watershed and creation of water divisions ((SAWPA), 2004). The populations of the Santa Ana Speckled Dace are highly effected by climatic events such as fire and floods. In 1995, the Santa Ana Speckled Dace was listed as a species of special concern by the California Department of Fish and Wildlife. Then in 1998 it was listed as a species of concern by the United States Forest Service. The Santa Ana Speckled Dace was not listed as a federally protected endangered species due to the lack of formal peer reviewed taxonomic description including a genetic description of the taxon and population level diversity (Moyle & Marchetti, 2006).

An excellent source of population genetic analyses is the highly polymorphic markers, microsatellites. Microsatellites are 1-6 base tandem repeats found in nuclear DNA. Currently, no such markers have been identified for the speckled dace. The objective of this project is to characterize microsatellite loci for

Rhinichthys osculus and then perform molecular analysis on the data acquired from a variety of populations across Southern California to examine gene flow and historic patterns of interbreeding among creeks. Research is currently being performed in the Molecular Ecology and Evolution lab of Dr. Tony Metcalf on the cyt b and d-loop regions of mitochondrial DNA (mtDNA); mtDNA is only inherited from the maternal lineage of an organism. Microsatellites, because they are located on nuclear DNA, represent both the maternal and paternal lineages of the specimen. With this data, we hope to gain a better understanding of the evolutionary histories and developments that have occurred among the local populations of *Rhinichthys osculus*.

Materials & Methods

In order to evaluate the genetic variation that exists between the various tributaries of the Santa Ana River Watershed, *Rhinichthys osculus* samples were collected, in collaboration with the United States Forest Service and California Department of Fish and Wildlife, from various sampling sites representing each of the local watershed tributaries, as well as neighboring watershed habitats for comparison (Figure 2).

Figure 2: Rhinichthys osculus California Range Map. Areas bordered in dark are known to contain Rhinichthys osculus within river drainages of that region. Proposed watershed sampling locations are denoted in red numbered circles. 1=Colorado River; 2=Santa Ana River; 3=San Gabriel River; 4=Santa Maria River; 5=San Luis Obispo River; 6=Owens River.

In addition, newly acquired samples will be collected using proper electroshocking technique under accordance with permits issued to the Metcalf Lab by the United States Forest Service. For each specimen that has been collected by the Metcalf Lab, GPS coordinates were taken from the sampling location on the designated tributary. A minimum of seven *R. osculus* samples were acquired from each tributary making sure that specimens were acquired from various locations in the tributary in order to evaluate genetic variation within the populations inhabiting the tributary. Each *R. osculus* sample underwent genomic DNA extraction using phenol-chloroform extraction methods utilizing phase lock gels (PLG) as set forth in Eppendorf's Phase Lock Gel Manual (Mouse Tail Genomic DNA Isolation Protocol). Genomic DNA extractions were then visualized using agarose gel electrophoresis. DNA concentrations were analyzed for all tissue extractions using spectrophotometry (A260/A280 and A234/A260). Thirty-three *Rhinichthys osculus* samples were used to characterize and identify microsatellite loci in conjunction with the Savannah River Ecology

Lab. Utilizing pair-end Illumina shotgun sequencing, microsatellite loci were identified for *R. osculus.* Using specific conditions and parameters in *PAL_FINDER_v0.02.03* (Castoe, et al., 2012), 48 microsatellite loci were identified and primers were designed. Forty-eight primer pairs were then tested for amplification and presence of polymorphisms using DNA obtained from eight individuals. PCR amplifications were performed in a 12.5 µL volume (10mM Tris pH 8.4, 50mM KCl, 25.0 µg/ml BSA, 0.4 µM unlabeled primer, 0.04µM tag labeled primer, 0.36µM universal dye-labeled primer, 3.0mM MgCl2, 0.8mM dNTPs, 0.5 units AmpliTaq Gold® Polymerase (Applied Biosystems), and 20ng DNA template) using an Applied Biosystems GeneAmp 9700. Loci were amplified using a touchdown thermal cycling program (Don, Cox, Wainwright, Baker, & Mattick, 1991)covering a 10°C span of annealing temperatures ranging between 65-55°C (TD65).

Touchdown cycling parameters consisted of an initial denaturation step of 5 min at 95°C followed by 20 cycles of 95°C for 30 s, 65°C (decreased 0.5°C per cycle) for 30 s, and 72°C for 30s; and 20 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. Loci that did not amplify with a touchdown PCR protocol were screened with the same PCR protocol but using a single annealing temperature of 65°C for all 40 cycles. PCR products were run on an ABI-3130xl sequencer and sized with Naurox size standard prepared as described in DeWoody et al. (2004). Results were analyzed using GeneMapper version 3.7 (Applied Biosystems). Twenty-three of the tested primer pairs amplified high quality PCR product that exhibited polymorphism (Taken from Nunziata, Lance, Jones, Nerkowski, & Metcalf, 2003).

All twenty-three polymorphic microsatellite loci have undergone amplification utilizing the Metcalf lab equipment, through the use of PCR and the corresponding unlabeled primer pairs. PCR conditions initially followed the protocols used in Girard and Angers (2006). Each 12.5µL PCR reaction consisted of 1.5mM of $MgCl_2$, 2.5mM of each dNTP, 0.2U of *Taq* polymerase, 1.25µL of 10x *Taq* polymerase buffer, 10pmol of each primer and 10ng of genomic DNA. The PCR program, utilizing BioRad C1000 Thermal Cycler, consisted of an initial denaturing temperature of 92°C for 30s, then 45 cycles of the following profile: 92°C for denaturation, 15 seconds at annealing temperature 65°C, and 5 seconds at 68°C. The final phase of the cycle is a 2 minute extension at 68°C. PCR amplicons were then visualized utilizing a 2% METAPHOR© gel. PCR analysis and examination of the amplified products are being further evaluated and analyzed on a 6.5% polyacrylamide gel using the LICOR model 4300 automated DNA analyzer.

PCR protocols, annealing temperatures and reagent concentrations are currently being adjusted to provide maximal optimization of the microsatellite loci. A microsatellite locus is considered optimal in agarose gel electrophoresis by a strong single or double band without the presence of stutter bands or other products. Each sample, with an amplified microsatellite locus, will be scored to determine base pair length and polymorphism using SAGA (LI-COR, INC.). Currently, each locus is being analyzed to determine its usefulness in analyzing genetic variation among and within populations of *R. osculus*. Various statistical programs will be utilized to examine multiple population genetic parameters.

Results

Thirty-three *Rhinichthys osculus* samples were used to characterize and identify the 23 polymorphic microsatellite loci at the Savannah River Ecology Lab. Table 1 represents all 23 polymorphic loci identified. When analyzing the repeats at each locus, they identified eight microsatellite loci with significant deviations from the Hardy-Weinberg expectations (linkage disequilibrium); Rhos 1, Rhos 8, Rhos 9, Rhos 21, Rhos 22, Rhos 25, Rhos 31 and Rhos 35 (Nunziata, Lance, Jones, Nerkowski, & Metcalf, 2003). In addition, 7-25 alleles were observed at each of the various loci.

Twenty-one of the twenty-three microsatellite loci successfully amplified under the protocols set forth by Girard and Angers (2006). Figure 3 illustrates a 2% metaphor gel for locus, Rhos 10.

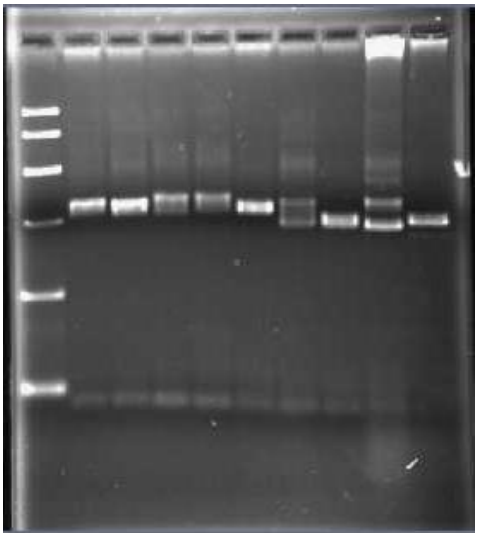

Figure 3: 2% metaphor gel for microsatellite locus, Rhos 10, for 9 different samples from various watersheds throughout California. Girard and Angers (2006)PCR protocol was utilized to examine microsatellite loci with unlabeled primers.

As seen in Figure 3, homozygotes and heterozygotes are observed, as well as various repeat numbers, which suggests genetic variation within the population for this particular locus. Each of the other microsatellite loci exhibited similar 2% metaphor gel images suggesting that the microsatellite loci that were identified were indeed polymorphic and useful in our genetic studies.

Discussion

This purpose of this study is to examine the biogeography of populations through the analysis of microsatellite loci that are less variable and more conserved, and comparing them to the Owens River, Central Coast and Colorado River populations of *Rhinichthys osculus*. The Southern California region has gone through extensive geological and climatal changes throughout its history altering the landscape and topography of the region including pluvial and arid time intervals (Colburn, 2006). Each of these events could alter the evolutionary history of an organism and determine the levels of dispersal as well as gene flow that can exist between populations. Freshwater tributaries within the Santa Ana River Watershed have also undergone such events that will lead to the divergence of aquatic populations. *R. osculus* was once able to freely inhabit the free flowing and perennial rivers within the Santa Ana River Watershed but due to changes in climate and geology and more recent anthropogenic causes in the last century, their habitat became discontinuous and fragmented. Currently, due to the discontinuous and fragmented habitats of *Rhinichthys osculus*, the degree of gene flow between populations is very limited, if it exists at all (Cornelius, 1969; Oakey, 2004).

In addition, this study, in conjunction with the Savannah River Ecology Lab has developed a library of microsatellite loci for *Rhinichthys osculus.* Twenty-three polymorphic microsatellite loci (Table 1) have been identified that may be useful in our studies of *Rhinichthys osculus* populations. This research project is ongoing. Currently over 150 samples of *Rhinichthys osculus* are being genotyped for fifteen different microsatellite loci. Upon analysis of the data we will examine the relationships between the populations inhabiting the tributaries of the Santa Ana Watershed system as well as the San Gabriel Watershed system. Through our analysis we hope to gain a better understanding of the molecular evolution, phylogeography, population genetics and conservation of the local populations of the Santa Ana Speckled Dace. This data, along with the mtDNA work and other nuclear DNA work that is being performed in the Metcalf lab, will allow government agencies to determine if conservation management strategies are necessary.

Locus	Repeat motif	Size (bp)
Rhos1	AATG	228-236
Rhos3	ATCT	268-436
Rhos5	ATCT	237-313
Rhos8	AAAG	173-281
Rhos9	ATCT	145-185
Rhos10	ATCT	305-377
Rhos14	ATCT	262-322
Rhos16	AATG	168-312
Rhos18	ATCT	252-360
Rhos20	ATCT	246-366
Rhos21	AAAG	162-246
Rhos22	ATCT	365-413
Rhos23	ATCT	218-358
Rhos25	AAAG	126-214
Rhos26	ATGG	103-163
Rhos27	ATCT	274-330
Rhos29	ATCT	204-264
Rhos31	ATCT	173-305
Rhos33	ATCT	324-372
Rhos35	ATCT	302-354
Rhos36	AAAG	144-204
Rhos42	AAAC	140-191
Rhos43	AAC	256-274

Table 1: Microsatellite loci and primer information developed in conjunction with the Savannah River Ecology Lab. The size (bp) indicates the range of observed alleles in base pairs and includes the length of the CAG tag, * indicates CAG tag (5'-CAGTCGGGCGTCATCA-3') label (modified from Nunziata et al. 2013).

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