Final Report

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Population genetics of the threatened South Texas Siren (large form SP1)

Prepared by: Richard J. Kline & Lucia B. Carreon
Principal Investigators

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Abstract

The South Texas Siren, its present distribution and population status are not well understood. Field surveys for sirens were conducted at 9 geographic locations and 22 separate water bodies within Cameron, Hidalgo and Kleberg counties using baited minnow traps. Sirens were found in 12 water bodies during the course of the study. A photo recognition program was successfully used to identify spotting patterns in the South Texas Siren and showed promise in future mark-recapture studies. Primers for CO1 and 16S mitochondrial DNA markers were developed for sirens and showed differences between published sequences. A microsatellite library for the South Texas Siren was developed, and primers were designed and tested resulting in eight identified regions that were polymorphic and can be used for population studies of this species. This survey and analysis has provided additional tools for the management and conservation of sirens in South Texas resacas, ponds, and water bodies.

Introduction

At least one species of siren is endemic to South Texas, inhabiting shallow bodies of water such as those commonly known as "resacas" and wetlands (NatureServe, 2013). It is unclear where the boundaries are for the South Texas Siren (large form SP1) as defined by TPWD. Reports indicate that sirens may have occurred as far north as San Patricio and Jim Wells counties (NatureServe, 2013). The South Texas Siren has been considered threatened since 2003 by TPWD (TPWD www.twpd.state.tx) and is classified as vulnerable due to numerous anthropogenic factors making it susceptible to extirpation. Presently, there is no consensus on the species identification of the siren species that inhabit South Texas or their overall population status. In the past, a subspecies of the lesser siren, *Siren intermedia texana* was referenced by TPWD as the one that occurred in South Texas (Villela and Brandon, 1992). The lesser siren has a much broader historic distribution, around the Atlantic and Gulf Coastal Plain from Virginia to Mexico (Lannoo, 2005). Villela and Brandon (1992) proposed that 2 species, *S. intermedia* and *S. lacertina*, occurred sympatrically in South Texas and Mexico by morphological comparison of museum voucher specimens. However, the results from a recent phylogenetic study indicate that there are two distinct species in Texas, but evidence of sympatry or population status is unclear (Corey Roelke, UT-Arlington, pers. comm.). For this reason, intensive local sampling, data on molecular phylogenetics, distribution, and population genetic structure of the siren species found in South Texas would aid in resolving long-standing questions about the status of the South Texas Siren(s).

The South Texas siren and other amphibians that live in the Rio Grande Delta the four ancient distributaries of the Rio Grande, or the numerous resacas (ox-bow lakes), are currently at risk from many sources of habitat degradation such as dredging and sand mining. These actions,

along with urban development, agricultural activities, and reduced water flows have contributed to habitat degradation and perturbation. Furthermore, alteration of flood and dry-out cycles of the local resacas, and the limitation of the meander of the Rio Grande connectivity, has greatly reduced the potential for dispersal of this species from historic levels; hence there is an urgent need for a study at the population level on sirens and the development of tools to accomplish this. The South Texas siren represents the unique diversity endemic to this small portion of Texas. Moreover, sirens, as with other amphibians, can serve as indicator species of the health of these water bodies—a resource worth billions of dollars to the humans that utilize them.

Conservation of threatened or endangered species relies primarily on *in situ* protection practices (Seddon *et al*. 2007); to accomplish a successful conservation effort it is best to have extensive knowledge about the species in question. The utilization of molecular markers such as microsatellites and mitochondrial DNA (Hagerty *et al*. 2008) can provide such information. Molecular, nuclear, and mitochondrial markers can yield valuable information that has not been available for this particular species, such as: evidence of sympatry between more than one species, gene flow, inbreeding or hybridization between species, and the extent of gene flow. Recent advances in photo-documentation of salamanders (Bendik *et al*., 2013) in conjunction with these methods can control for repeated sampling of the same individuals for DNA analysis, and may provide information on habitat connectivity. With the global decline in amphibian species and loss of essential aquatic habitats, application of molecular techniques is necessary to aid in decisions of conservation status by correctly identifying species and documenting population state (Lannoo, 2005). Given the lack of information regarding these species at the population level, it is imperative that we develop the tools to assess their population dynamics to establish the conservation status of this important species.

Objectives

The objective of this research was to collect South Texas sirens in their reported historic habitat range and to develop mitochondrial and genomic microsatellite markers for the study of their population genetic structure. The development of capture and photo analysis techniques for mark-recapture studies and documentation of co-occurring amphibian species was also conducted. The specific aims of this study were 1) to use the South Texas Siren microsatellite library to characterize population patterns; 2) to create a photo library database for the South Texas Sirens captured during the study and analyze the spot pattern using the Wild-ID program; 3) To assess if any sympatric siren species were present in the samples collected in this study using mitochondrial DNA sequences; and 4) to test the microsatellite regions in the South Texas siren for evidence of allele polymorphisms and similarity in the samples collected in relation to location captured.

Materials and Methods

Field collections

Trapping for Sirens was conducted in South Texas resacas and ponds in Cameron, Hidalgo and Kleberg counties. Baited minnow traps were placed at each location overnight and checked in the early morning (Sorensen, 2003). Captured sirens were photographed, measured, weighed, sampled for DNA from the caudal fin, then released. Location and local water parameters were recorded, including water temperature, pH, conductivity, and dissolved oxygen. Habitat features such as vegetation type and cover, sediment type, degree of urbanization were also recorded.

Photo-analysis

A photo database of 28 field collected sirens was compiled. Photos were taken with an iPhone, and later with a Canon Rebel T3i with 100mm macro lens and flash. Photos were taken with the sirens out of the water and submerged below the surface to compare the best photo conditions. A total of 289 photos were analyzed for different angles and exposure settings. Fifty-nine pictures were selected for testing in the Wild-ID program following publisher's instructions with repeated photos of some individuals used as test of the capability of Wild ID in the identification of individual sirens. Photos were cropped to use only the head region and to eliminate the gills. Photos were rotated so that the head was facing down. Both white and black background holding containers were used.

Mitochondrial DNA sequences for CO1 and 16S

Four sets of published primer sequences for isolating mitochondrial regions were used on extracted siren DNA. These primer sequences were unsuccessful for the siren samples used, therefore published DNA sequences for *Siren lacertina* and *Siren intermedia* were used to design new primer sets specific for CO1/ NADH and 16S regions. Four primer sets were designed and PCR conditions optimized to yield one fragment of the expected size. These fragments were purified then sequenced. Sirens were analyzed at different regions of their mitochondrial genome for 16S and cytochrome oxidase 1 to discriminate between other species that might be present in the area using published sequences in GenBank for *Siren intermedia* and *Siren lacertina*.

Microsatellite library development

The microsatellite library was designed following the protocol described in Fischer and Bachman (1998) and Hamilton *et al* (1999). After DNA enrichment, hybridization and cloning, 236 inserts were sequenced to find the genetic regions with the microsatellite (i.e. variable tandem repeat) regions and flanking regions appropriate for primer design. Thirty five sequences were selected as candidates and primer sets were designed using Primer3Plus (Untergasser and Nijveen, 2007). Four different primer sets per sequence were screened for successful amplification using PCR with a temperature gradient to select the best annealing temperature for each primer set. The

primer sets with the best results at the optimized temperature were selected for testing with eight siren samples across the geographic sampling range for evidence of polymorphism.

Results and Discussion

Of the 31 sirens caught during this research, all of them had the 36 costal grooves and rounded tails found in descriptions of *Siren lacertina*. They ranged in length from 240 mm to 510 mm and in weight from 35 g to 390 g (Figure 1). Of all the sites where sirens were captured, water temperature ranged from 26.4˚ C to 31.6˚ C, pH varied between 6.5 and 8.8, and water conductivity ranged from 8.95 to 1819. No differences were observed with regards to the water parameters measured or amphibians and insect species present between sites where sirens were captured and those where they were not. The characteristics that distinguished sites where sirens were captured were their rural location and that they had dried out in the recent past.

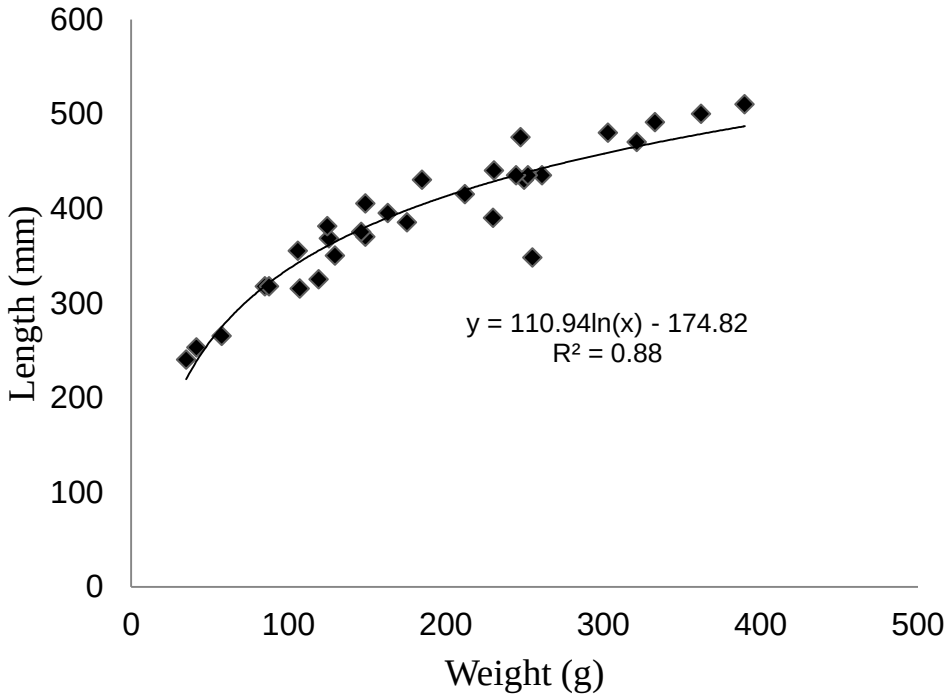

Figure 1. Length and weight relationship of sirens sampled in this study.

Photo analysis

A white rounded container with the siren submerged underwater provided the best holding conditions in terms of contrast and animal movement. Sirens would move continuously when out

of water but would remain relatively calm and stationary when submerged. In trials with rectangular containers, sirens would seek out a corner of the holding chamber making photography difficult. Due to turbidity, rinsing of the siren and filtration of the water is recommended. The Canon Rebel with 100mm lens, polarizer and slave flash provided the best photos; however, additional training is needed for personnel using the camera in comparison to relatively simple iPhone.

Repeated photos from the same individuals taken from different angles and lighting were correctly distinguished with the Wild ID program from the rest of the photos in the database with an average of 34.5% similarity score with a low of 8% and a high of 92%, indicating that a photo-based mark-recapture study is possible with the South Texas Siren. Interestingly, most individuals had 0% match score to others; however, some individuals from the same water bodies had low but detectable match scores (~4%), potentially indicating relatedness apparent from spotting patterns.

Mitochondrial DNA analysis

Fragment sequences from captured individuals were compared to published sequences listed in GenBank database. The CO1 sequence from sirens in this study was 99% similar to the published sequence for *S. lacertina* and 91% similar to *S. intermedia*. Interestingly, the 16S fragments were 98% similar to *S. intermedia* and 94% similar to *S. lacertina*, indicating some differences in the South Texas population (Table 1). Eight samples across the geographic range that were sequenced for CO1 and 16S were identical, indicating that these individuals were likely the same species of siren.

Table 1. Primers designed to successfully amplify CO1 and 16S mitochondrial DNA from the South Texas sirens captured in this study and sequence similarity values as compared to published sequences for *Siren lacertina* and *Siren intermedia*.

Locus	Primer sequence	Ta (°C)	Size (Bp)	*S. Lacertina*	*S. Intermedia*
Siren CO1	F: GTAAAACAAAATATAGTAATAATAGC	48	610	99%	91%
	R: TAGTCATCGGGTGATTATC				
Siren 16S	F: CGCCTCTTGCATTCCAAATA	55	505	94%	98%
	R: GTCCTGATCCAACATCGAGGT				

F Forward primer sequence, R reverse primer sequence, Ta annealing temperature, PCR conditions were 95°C for 2 min, 30 cycles of 95°C for 30 s, Ta for 30 s and 72°C for 30 s, followed by a final extension of 72°C for 5 min. Amplicon sizes are based on sequenced products. Compared GenBank sequences were *Siren lacertina* CO1 (AY916041.1), *Siren intermedia* CO1 (AY916040.1), *Siren lacertina* 16S (DQ283181.1), *Siren intermedia* 16S (Y10946.1).

Microsatellite library development

Eight microsatellite primer sets were selected due to the specificity and evidence of polymorphisms among the siren individuals tested. The primer sequences, fragment size ranges and optimal PCR conditions are listed in Table 2.

Table 2. Primers for polymorphic microsatellite loci from the South Texas siren based on 8 individuals.

Locus	Primer sequence		Motif	Ta (°C)	Range (Bp)
A78040-81	F:	TAGGGCACCTGTGCATAACA	AC^14	63	230-245
	R:	GCCCTTCAATCCGTAAGACA			
A78000-41	F:	CCTTTTCTCTGGGCTGACTG	CA^14	63	230-240
	R:	GGGTCTACAGGTGTGTGTGC			
A78012-53B	F:	ATTCACCCGCTCCCTCAC	CT^19	62	225-250
	R:	GGGAGTGTGTGCAGGTATGT			
A77974-15	F:	GGTGAAGGATTTCACCGTGT	CATA^6	60	240-255
	R:	TAAAGCTGGAAGCCGGAGTA			
A78012-53	F:	CACTCCCTCCCTCACTTTCT	CA^14...CT^19...CACA^7	50	370-390
	R:	TGGGAGTGTGTGCAGGTATG			
A77978-19B	F:	TGGGGGACTTCACATTTAACA	TC^10	56	145-160
	R:	CAGAGAGGTGGAGACCCAAG			
A78017-58	F:	AGAAGCACATGTGGTGTATGC	AC^12	54	375-390
	R:	GCGTGTAAGCGAGCATGTAT			
A77978-19A	F:	TGGGGGACTTCACATTTAACA	AC^19	60	250-260
	R:	CCAAGGAGAGTGGAGCAGAG			

F Forward primer sequence, R reverse primer sequence, Ta annealing temperature, PCR conditions were 95°C for 2 min, 30 cycles of 95°C for 30 s, Ta for 30 s and 72°C for 30 s, followed by a final extension of 72°C for 5 min. Amplicon sizes were visually analyzed on 6% polyacrylamide Megagel and reference ladder.

Conclusions and Recommendations

The array of eight polymorphic microsatellite regions identified in this study will be useful and future research assessing the population structure of the South Texas Siren should be conducted. Although sirens were not found in several water bodies in South Texas during this study, these cannot be ruled out as potential siren habitat without further sampling attempts. Future studies should focus on repeated and intensive sampling of water bodies to characterize habitat and

develop a habitat suitability index for the South Texas Siren. Differences identified in the mitochondrial DNA sequences are intriguing and should be investigated further by isolation and sequencing of larger regions of the mitochondrial genome.

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**In the report 12 sites are listed as containing sirens. However, sirens were collected on 12 separate occasions from 7 sites.

Site	Site #	Date	Siren collected	GPS	Trap hrs
The Nature Conservancy – Front Pond	1	9/11/2013	1 2 3	N: 25°51.264', W: 97°23.862'	17hrs 1 trap 1 trap w/sirens
The Nature Conservancy – Front Pond	2	9/27/2013	4 5 6	N: 25°51.264', W: 97°23.862'	3.5hrs 8 traps 3 traps w/sirens
TPWD – Pond 2	3	9/28/2013	7 8	N: 25°59.142', W: 97°31.867'	15hrs 6 traps 2 traps w/sirens
TPWD – Pond 3	4	9/28/2013	9	N: 25°59.166', W: 97°31.871'	15hrs 6 traps 1 trap w/siren
TPWD – Pond 2	5	10/4/2013	10	N: 25°59.142', W: 97°31.867'	16hrs 9 traps 1 trap w/sirens
TPWD – Pond 3	6	10/4/2013	11	N: 25°59.166', W: 97°31.871'	16hrs 9 traps 1 trap w/sirens
Sabal Palm Sanctuary – South Resaca	7	10/11/2013	12 13 14	N: 25°50.941', W: 097°25.179'	17hrs 12 traps set 3 traps w/ sirens
Sabal Palm Sanctuary – Main Resaca	8	10/11/2013	15 16 17 18	N: 25°50.949', W: 097°25.159'	17hrs 12 traps set 2 traps w/ sirens
Sabal Palm Sanctuary – Main	9	10/17/2013	19 20 21 22 23 24	N 25°51.050', W 097°25.168'	16hrs 16 traps set 5 traps w/ sirens
Sabal Palm Sanctuary – Walkway	10	10/19/2013	25 26 27	N 25°51.109', W 097°25.229'	16 hrs 5 traps 3 traps w/sirens
Sabal Palm Sanctuary - Main	11	10/19/2013	28 29	N 25°50.963' W 097°25.144'	16 hrs 5 traps 3 traps w/sirens
Remberto Property – Pond 2	12	11/21/2013	30	N 27°23.522', W 097°50.783'	24hrs 9 traps 1 trap w/siren
The Nature Conservancy – Front Pond	13	12/4/2013	31	N: 25°51.264', W: 97°23.862'	FOUND DEAD

Siren Captures - Site Characterization – Vegetation/measured indices

- The Nature Conservancy
    - Front pond – man made cement pond, 3 sides with vertical cement walls, one with gradual incline to grass area. Thick water plants - sedge species, water lilies, tall reeds.
    - Back Resaca – Very thick guinea grass and Georgia cane.
- Sabal Palm Sanctuary
    - Small South End – Extremely dense patches or guinea grass around edges of the Resaca, especially in very shallow areas. Resaca bordered with tall Georgia Cane and Tepehuaje trees.
    - Bird Blind Resaca – Thick Guinea grass around edges of Resaca. Bordered with Tepehuaje, Georgia cane, and thorny plants. The thorny plants overhang on the steeper sides of the banks. Muddy banks.
    - Walkover Resaca – Shallow water, recently re-flooded (2014) mesquite trees, retama, and huisache trees still living in the water submerged. Decaying leaves and grass and other vegetation on bottom.
- TPWD Fish Hatchery
    - Pond 1 – Very shallow and narrow pond. Lined with large oaks, and sparse guinea grass.
    - Pond 2 – Lined with guinea grass and sparse thorny trees.
    - Pond 3 – Extremely thick guinea grass, and dense, thorny trees overhanging into pond.
    - Pond 4 – Large pine trees around border, thick guinea grass, and thorny vegetation.
- Remberto Property
    - Pond 1 – Small area, covered in duckweed, algae, grasses, and small thorn trees. Gradual incline into pond. Extremely sticky mud.
    - Pond 2 – Edges covered in thick grasses, few small thorned plants. Extremely sticky mud.

Site	Date	Dissolved Oxygen (mg/L)	pH	Conductivity (µS)	Water temp. (C)
The Nature Conservancy - Pond	9/11/2013	N.D.	8	532	N.D.
The Nature Conservancy - Pond	9/27/2013	N.D.	7.2	463	N.D.
TPWD – Pond 2	9/28/2013	3.0 (mg/L)	6.96	1127	N.D.
TPWD – Pond 3	9/28/2013	N.D.	N.D.	N.D.	N.D.
TPWD – Pond 2	10/4/2013	4.6 (mg/L)	7.53	1146	N.D.
TPWD – Pond 3	10/4/2013	N.D.	7.80	1540	N.D.
Sabal Palms – South Resaca	10/11/2013	6.3 mg/l	N.D.	1003	26.7
Sabal Palms – Main Resaca	10/11/2013	1.5 (mg/L)	N.D.	950	26.7
Sabal Palms –	10/17/2013	4.4 (mg/L)	7.51	1024	26.8

Main					
Sabal Palms – Main	10/19/2013	6.8 (mg/L)	8.2	1033	27.2
Sabal Palms - Walkway	10/19/2013	4.5 (mg/L)	7.86	995	28.3
Remberto Property – Pond 2	11/21/2013	7.5 (mg/L)	7.88	85	31.6