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Mitochondrial DNA and Microsatellite Genetic Variation of Dollar Sunfish (Lepomis marginatus)

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Eastern Illinois University

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Mitochondrial DNA and Microsatellite Genetic Variation

of Dollar Sunfish (Lepomis marginatus)

(TITLE)

BY

Nancy A. Schable

THESIS

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Abstract

Dollar sunfish (*Lepomis marginatus*) at the Savannah River Site were sampled from two radiocesium contaminated ponds with a history of thermal elevation and from reference ponds with no history of radioactive or thermal contamination. Fluorescent sequencing of a portion of the mitochondrial control region showed no genetic variation within or between populations. Centrarchidae microsatellite loci Lma 120 (6 alleles, N=124 from 5 populations), Lma 20 (10 alleles, N=37 from 3 populations) and Rb 7 (14 alleles, N=17 from 2 populations) were variable for dollar sunfish. An additional 9 microsatellite primer pairs were designed for dollar sunfish. Testing of the designed primers on ≥20 individuals from 5 populations showed these loci have a high number of alleles ranging from 4 to 14 (mean = 8.6) and observed heterozygosities ranged from 0.500 to 0.857 (mean heterozygosity = 0.6337). Age analysis showed most individuals were 1-3 years of age. Only 14 individuals from two contaminated sites, Pond C and the West Arm of PAR, had levels of radiocesium in their muscle tissue above the detection limit. Tissue samples need to be re-measured using larger quantities of tissue for more accurate results in order to correlate radiocesium concentration to fish age. Overall, no comparison of genetic variation could be made for contaminated and uncontaminated ponds using mitochondrial DNA. However, new microsatellite primers for dollar sunfish have been designed for future analyses, and preliminary results indicate that they will be adequate for examining genetic variation for this species.
I respectfully dedicate this thesis to my loving parents

James and Janet Schable.
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**Table of Contents**

Abstract ....................................................................................... 1  
Dedication..................................................................................... 2  
Acknowledgements........................................................................ 3  
Introduction and Literature Review....................................................... 6  
Study Site.................................................................................... 11  
Materials and Methods.................................................................... 12  
  Collection........................................................................... 12  
  DNA Isolation....................................................................... 13  
  Mitochondrial DNA Amplification and Sequencing ......................... 14  
Centrarchidae Microsatellite Primers for Dollar Sunfish ...................... 15  
Microsatellite Primer Development for Dollar Sunfish......................... 16  
Aging Procedure.......................................................................... 23  
Radiocesium Analysis.................................................................... 23  
Results........................................................................................ 24  
  Mitochondrial Control Region................................................... 24  
  Centrarchidae Microsatellites for Dollar Sunfish ....................... 25  
  Microsatellite Development Results....................................... 25  
  Aging and Radiocesium Results........................................... 26  
Discussion................................................................................... 26  
Literature Cited.......................................................................... 32  
Tables......................................................................................... 40  
  Centrarchidae Microsatellite Primers................................. 40
Oligo Mix Information .......................................................... 41
New Dollar Sunfish Microsatellite Primers .................................. 42
Aging Data ........................................................................ 43
Figure Legend ............................................................................. 44
Savannah River Site ................................................................. 45
Super SNX Linker ...................................................................... 46
Dollar Sunfish Mitochondrial Control Region Sequence .................. 47
Age Trends ......................................................................... 48
Age Composition ....................................................................... 49
I. Introduction and Literature Review

Nuclear reactor operations such as those performed at the Savannah River Site (SRS) use fission, the splitting of nuclei, to start a chain reaction that produces energy, heat, and radioactive wastes. Water is used to cool the nuclear reactor core and is released back into the environment in the form of thermal effluent. The thermal environment of the entire cooling reservoir may be altered as a result (Enger & Smith 1998).

Thermal influences created by nuclear reactors have been demonstrated to influence life history strategies of organisms. Shuter et al. (1985) described how smallmouth bass (Micropterus dolomieui) exposed to thermal effluent demonstrate changes such as early spawning, increased egg and larval mortality, increased growth of surviving juveniles, and decreased winter mortality. Year classes were expected to either “boom” or “bust” based on the thermal regime dictated by reactor runs (Shuter et al. 1985). Wellborn and Robinson (1996) examined macroinvertebrate population responses to thermal alteration of a Texas pond in comparison to a main reservoir (which experienced no artificial temperature increase). They found that macroinvertebrate population dynamics in a thermally elevated reservoir were similar to those described for smallmouth bass with an overall lower macroinvertebrate abundance and reduced taxonomic diversity.

In addition to increased thermal input, there have been several incidences of radioactive leaks associated with nuclear reactor production facilities. Chernobyl and Three Mile Island are well known for accidents that released radioactive contaminants into the environment (Enger & Smith 1998). The Savannah River Site near Aiken, South
Carolina, is a less commonly known location at which there has been radioactive contamination of sediments in several cooling reservoirs associated with nuclear reactors that produced materials for nuclear weapons. Radiocesium ($^{137}\text{Cs}$) is one radioactive waste created by nuclear reactors, and it is of great interest due to its ca. 30-year half-life, ease of detection and its ability to accumulate in the body tissue of exposed organisms. This is of ecological importance because radiocesium emits gamma radiation, which has been shown to cause damage at organismal, organ, tissue, and cellular levels (Enger & Smith 1998). Gamma radiation also is capable of causing damage at the molecular level creating DNA mutations that may result in abnormal offspring or cancer (Enger & Smith 1998; Griffiths et al. 1996). Muller (1927) was one of the earliest researchers to document the harmful effects of radiation exposure by examining the genetics of fruit flies (Drosophila melanogaster) exposed to X-rays. More recent studies such as those performed by Dubrova and Plumb (2002) and Yauk et al. (2002) examine more closely the harmful effects of ionizing radiation on DNA.

Thermal effluent and radioactive contamination play an important role in altering an ecosystem and its associated organisms. One may predict that when organisms are subjected to extreme environmental conditions caused by perturbations, differential reproduction and survival of individuals within that population will occur which may affect genetic variation (Krebs & Davies 1981). A method by which genetic variation may be reduced in these extreme environments is a sudden decrease in population size, also referred to as a bottleneck (Avise 1994). After a bottleneck event, the effective population is small and additional genetic variation is likely to be lost due to genetic drift, or chance fluctuations in allele frequency as a result of random sampling among gametes.
Hartl 1988). The inability to find a mate is an example by which genetic variation may be lost from a population due to chance (Hartl 1988).

Patterns of genetic variation among populations in polluted environments may be examined using a variety of molecular techniques. Mitochondrial DNA (mtDNA) is commonly used to examine genetic variation. In particular, the mtDNA control region is a noncoding segment that often functions as the initiation site for heavy-strand replication and the promoter for heavy and light strand transcription (Clayton 1984). Because the function of the control region is more highly associated with cloverleaf structure conservation rather than a particular sequence, it often contains a great deal of sequence variation among individuals and species (Brown 1986; Kimura & Ohta 1974). It has been suggested that mtDNA has a high mutation rate as a result of two possible factors. First, rate of nucleotide misincorporation is higher for mtDNA than for single copy nuclear DNA (Hartl 1988). Second, replication errors often remain uncorrected because mtDNA polymerase has inefficient proofreading functions in comparison to nuclear DNA polymerase (Hartl 1988; Wilson et al. 1985). Brown et al. (1993) found the white sturgeon (Acipenser transmontanus) hypervariable region had a four to five times higher substitution rate than other areas of the mtDNA. Using restriction enzyme studies, the average percent of nucleotide differences reported were 2.27% in the control region versus 0.54% in the whole mtDNA. Estimates of the rate of substitution in the human control region ranged between 2.8 and 5.0 times higher than the rate for other areas of the mitochondrial genome (Baker & Marshall 1997). Furthermore, in comparison to single copy nuclear DNA, mtDNA has a maternal haploid mode of inheritance resulting in a rapid rate of evolution and extensive intraspecific polymorphism (Avise et al. 1987;
Brown et al. 1979). Birky et al. (1983) showed that when male and female dispersal are equal, mtDNA geneflow is one-fourth that for the nuclear genome. A culmination of these features makes mtDNA an ideal indicator of population subdivisions, bottleneck events, and genetic drift (Chapman et al. 1982; Wilson et al. 1985). Methods commonly used to examine mtDNA have included restriction endonuclease mapping (Brown et al. 1979) and radiolabeled mtDNA probing (Mangini et al. 1985). More recent techniques for mtDNA analysis involve sequencing control region segments to examine base substitutions as an indicator of genetic variation patterns of organisms in response to environmental conditions (Finnerty & Block 1992; Ong et al. 1996). Although mtDNA sequencing is a very popular method of examining genetic variation patterns, other analysis tools may prove useful as well.

Microsatellite analysis is another molecular approach commonly used to examine genetic variation. Microsatellites are sequences made up of a single sequence motif, no more than six bases long, that is tandemly repeated, and that is arranged head-to-tail without interruption by any other base or motif (Hancock 1999; Tautz 1989). Microsatellites are a popular tool to use for genetic analysis because of several features. First, primers are highly specific because the microsatellite flanking region is usually single copy nuclear DNA (Litt & Luty 1989; Tautz 1989; Weber & May 1989). This feature results in a high probability that primers will be specific so that only amplification of the target gene will occur. Second, microsatellite repeats are small, allowing for easy amplification using the Polymerase Chain Reaction (PCR) (Litt & Luty 1989; Tautz 1989; Weber & May 1989). Third, microsatellites have a high mutation rate ($10^{-3}$ to $10^{-4}$ events per locus per generation for mice and humans) in comparison to point mutations.
(10⁹ to 10¹⁰) (Dallas 1992; Hancock 1999; Weber & Wong 1993). This lends to microsatellite loci having high levels of polymorphism (Tautz 1989; Weber & May 1989). Fourth, microsatellite loci mutate by gaining or losing repeat units allowing for detection of different alleles using electrophoretic techniques to examine length variants (Tautz 1989; Weber & May 1989). Finally, semi-automated genotyping applications for microsatellites include analysis at a variety of levels including determining gender, examining relatedness and parentage, examining genetic structure of populations, and comparisons among species (McDonald & Potts 1997; Ziegle et al. 1992). Microsatellite analysis of dollar sunfish may prove useful for examining patterns of genetic variation among populations of fish within and between environments with a history of thermal and radioactive contamination.

The purpose of this study was to examine genetic variation of dollar sunfish (Lepomis marginatus) exposed to thermal effluent and radiocesium. Dollar sunfish are ideal subjects for this study because they are small (approximate length 12cm), abundant in the southeast U.S. Atlantic Coast drainages, feed on insect larvae which often grow in sediments, nest on the sediment, and are unlikely to have altered patterns of genetic variation due to stocking for sport fishing (Page & Burr 1991). Thus, the objectives of this study were to:

1. Use mtDNA to examine genetic variation of dollar sunfish living in ¹³⁷Cs contaminated ponds with a history of thermal elevation and to compare this variation to dollar sunfish living in reference ponds.
2. Test already developed Centrarchidae microsatellite primers for polymorphism in dollar sunfish.
3. Develop microsatellite primers specifically for dollar sunfish.

4. Measure $^{137}$Cs content of dollar sunfish and analyze ages of dollar sunfish from contaminated and uncontaminated sites to determine if $^{137}$Cs bioaccumulates over the life of the fish.

It was hypothesized that the amount of genetic variation will differ between control populations and populations exposed to thermal effluent and radiocesium. It was also hypothesized that radiocesium levels will be undetectable in individuals from uncontaminated ponds whereas individuals exposed to contaminants will have detectable $^{137}$Cs levels that increase as a function of age.

II. Study Area

The Savannah River Site (SRS), located in west-central (Aiken and Barnwell counties) South Carolina, is a 780-km$^2$ former nuclear materials production and research facility administered by the U.S. Department of Energy (Figure 1) (Kennamer et al. 1993). Pond C (0.557 km$^2$) and Par Pond (8.724 km$^2$) are former cooling impoundments at the SRS. The North Arm of Par Pond received thermal effluent and periodic $^{137}$Cs contamination between 1954 and 1964 (Kennamer et al. 1993). Pond C and the Hot Arm of Par received thermal effluent between 1954 and 1987 and periodic $^{137}$Cs contamination between 1954 and 1964 (Brandt 1991; Kennamer et al. 1998). The West Arm of Par received no direct emissions of thermally elevated or $^{137}$Cs contaminated water (Kennamer et al. 1998). However, water movements, high temperature and natural processes created a dynamic environment that caused contaminated sediments to become suspended and move throughout the cooling system. Brisbin et al. (1973) described Par...
Pond as having a spatial mosaic of $^{137}$Cs contamination levels. Sediment samples from Par Pond were previously analyzed for $^{137}$Cs concentration, and results showed mean concentrations were as follows: North Arm 1.003 Bq/cm$^2$, Hot Arm 3.126 Bq/cm$^2$, and West Arm 0.165 Bq/cm$^2$. Several uncontaminated aquatic ecosystems on the SRS were sampled to serve as reference sites. They were Dicks Pond (0.010 km$^2$), Fire Pond (0.009 km$^2$), and Wetland 41 (0.006 km$^2$).

III. Materials and Methods

Collection

Twenty dollar sunfish were collected from 7 locations (4 contaminated and 3 reference) at the Savannah River Site. Contaminated sites were Pond C and the North, Hot and West Arms of Par Pond. Fire Pond, Dicks Pond and Wetland 41 were the reference sites.

Fish were captured using round ¼” mesh galvanized steel wire minnow traps (dimensions: 9”x17½”) (Memphis Net & Twine Co., Inc., Stock #G40M) set parallel to the shoreline in vegetated areas at approximately 30-100cm depth. Traps were checked once daily. Upon capture, the dollar sunfish were placed on ice for transport back to the lab for processing. Gill filaments were removed, placed in 400µL Tris High EDTA buffer (THE: 10mM Tris pH=8.0, 100mM EDTA, 1% SDS) and stored at −70°C. Otoliths were removed and placed in glycerol for aging. Finally, caudal peduncles were removed and placed in scintillation vials for radiocesium analysis. Fish, gill, and
peduncle samples were stored at -70°C, and otoliths were stored at room temperature until analysis.

**DNA Isolation**

Gill tissue was digested by combining 10µl Proteinase K (20mg/ml), and 5µl Rnase (10mg/ml) with 0.1 to 0.25 grams of gill in 400µl THE. Heart or muscle tissue was substituted when gill tissue DNA extraction was unsuccessful. The mixture was incubated on a rotator at 50°C for two hours or until digestion was complete. DNA extraction was performed using a protocol derived from Carter and Milton (1993). Specifically, 200µl of the tissue digestion was mixed with 800µl of Guanidine Thiocyanate (GuSCN) Extraction Buffer (4.06M GuSCN, 50mM MOPSO pH 6.5, 1% Triton X-100, 10mM EDTA) and 75µl of diatomaceous earth (50% in equal volume of deionized water). The mixture was incubated on a rotator at 50°C for 2 hours. Samples were centrifuged at high speed for 2 minutes and the supernatant discarded. The mud pellet was washed twice by adding 1ml of ice-cold 70% ethanol, centrifuging for 2 minutes, and discarding the supernatant. The diatomaceous earth pellet was dried at low heat and resuspended in 125µl Tris Low EDTA (TLE: 10mM Tris pH 8.0, 0.2mM EDTA pH 8.0). The suspension was centrifuged at high speed for 2 minutes. The DNA-containing supernatant was removed and placed in a microcentrifuge tube. Extracted DNA and mud pellets were stored at -20°C.

Chelex extraction modified from Walsh et al. (1991) was performed on heart tissue from Wetland 41 individuals due to difficulty obtaining DNA using diatomaceous earth extraction. Approximately 0.1 to 0.25 grams of heart tissue were added to 200µl of...
5% Chelex Extraction Solution (0.01g Chelex, 0.2% SDS, 10mM Tris pH 8, and 0.5mM EDTA pH 8.0). Next, 2µl of Proteinase K (20mg/ml) was added to each sample. They were heated at 65° and shaken every 15 minutes until the tissue was digested. Samples were then heated to 95°C for 5 minutes and immediately refrigerated. DNA was contained in the supernatant and diluted 1:19 before use as PCR template. Chelex DNA extractions were stored at 4°C. Stored at this temperature, chelex-extracted DNA was successfully amplified up to 45 days later.

Mitochondrial DNA Amplification and Sequencing

The left domain of the mtDNA control region was amplified using universal fish primers L15998-Pro (5’- TAC CC AAA CTC CCA AAG CTA-3’ (Bremer et al. 1996) and LMB-H396 (5’-AGG AAC CAG ATG CCA GGA ATA G-3’ T. Glenn unpublished). DNA from Wetland 41 individuals was amplified using primers L15998-Pro and CSBDH (5’-TGA ATT AGG AAC CAG ATG CCA G-3’ (Alvarado Bremer et al. 1996). Primer CSBDH created a slightly larger product providing higher quality annealing site for primer LMB-H396 during the sequencing reaction. Final concentrations of PCR reagents for a 25 µl reaction were 20 mM Tris pH=8.4, 50 mM KCl, 2.50 µg/ml BSA, 0.5 µM forward primer, 0.5 µM reverse primer, 1 mM MgCl₂, 150 µM dNTPs, 0.1 unit Taq DNA Polymerase isolated according to method of Pluthero (1993), and 50ng of DNA. A touchdown thermal cycling program (Don et al. 1991), was used with annealing temperatures between 65.0°C and 55.0°C decreasing 0.5°C per cycle. Cycling parameters were 5 cycles of 96°C for 20 sec, 65°C for 30 sec, and 72° for
1 min; 21 cycles of 96°C for 30 sec, 65°C minus 0.5°C per cycle for 30 sec, and 72°C for 1 min; and lastly 10 cycles of 96°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min.

Sequencing reactions were performed on the amplified mtDNA fragments in 10μl volumes. Ingredients included 50ng of PCR template, 0.33μM primer, and big dye terminators using ABI specifications except that the terminator mix was diluted 1:1 with halfBD™ (GENPAK LTD., Stony Brook, New York). Thermal cycling parameters were 40 cycles of 95°C for 20 sec, 50°C for 15 sec, and 60°C for 1 min. Sequence reactions were precipitated by adding 1μl of 1.5M Sodium Acetate (pH 7.5) + 250mM EDTA (pH 8.0) and 40μl of 95% ethanol. The mixture was mixed by inversion several times and incubated at -20°C for 20 minutes. The samples were centrifuged at 1,500xG for 45 minutes, and the supernatant was spun off of the sequence reaction pellet at 300xG for 1 minute. Dried sequence reactions were stored at -20°C until run on an ABI 377 automated sequencer. Sequence chromatograms were imported into Sequencher 4.1 (Gene Codes, Ann Arbor, Michigan, USA) where they were edited and assembled. The sequences were examined for presence of different haplotypes within and between populations.

**Centrarchidae Microsatellite Primers for Dollar Sunfish**

Several microsatellite loci designed for other members of the Centrarchidae family were tested on dollar sunfish (Table 1). Two of those loci, RB 7 and RB 20 were designed for redbreast sunfish (*Lepomis auritus*) (DeWoody *et al.* 1998). Lma 12, Lma 20, Lma 21, Lma 29, Lma 116, Lma 120, Lma 121, and Lma 124 were designed for bluegill (*Lepomis macrochirus*) (Colbourne *et al.* 1996; Neff *et al.* 1999). Lastly, MS 19
and MS 21 were designed for largemouth bass (*Micropterus salmoides*) (DeWoody *et al.* 2000). PCR final concentrations for a 25µL reaction were 10 mM Tris pH 8.4, 50 mM KCl, 0.25 µM forward primer, 0.25µM reverse primer, 1.5 mM MgCl₂, 1.5 mM dNTPs, 0.5 units Taq DNA Polymerase, and 30ng DNA template. Final concentrations for locus Rb 7 included 5% DMSO and 2.5µg/ml BSA. Primers were tested using touchdown thermal cycling programs as previously described encompassing a 10° span of annealing temperatures ranging between 65-55°C, 60-50°C or 55-45°C. Cycling parameters were: 5 cycles of 96°C for 20 sec, the highest annealing temperature for 30 sec, and 72°C for 1 min; 21 cycles of 96°C for 30 sec, the highest annealing temperature minus 0.5°C per cycle for 30 sec, and 72°C for 1 min; and lastly 10 cycles of 96°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min. Product was initially screened on a 1.5% agarose gel with ethidium bromide stain. Samples that appeared to amplify successfully were run on a 7% polyacrylamide gel to examine for polymorphism. Loci Rb7, Lma120, MS19, and MS20 were run with CXR (Promega, Madison, WI) genSIZE T350, or T500 Rox internal fluorescent ladder (Genpak, St. James, NY) on Perkin Elmer ABI 377 Automated Sequencer using GeneScan v3.1. Chromatograms were imported into Genotyper v2.5 (PE Applied Biosystems) to determine number of alleles per individual and allele size. Genepop 3.1c was used to examine observed heterozygosity among the individuals sampled (note that individuals were from various populations).

*Microsatellite Primer Development for Dollar Sunfish*

A restriction enzyme digest was performed on Dollar Sunfish diatomaceous earth DNA extraction by combining 5.0µl New England Biolab (NEB) Buffer 2 (provided by
manufacturer with purchase of Rsa I and Hae III enzymes), 0.5µl 100xBSA (Bovine Serum Albumin), 1.00µl Rsa I (NEB catalog #R0167S) or Hae III (NEB catalog #R0108S), 2.5µl H2O, and 40.0µl concentrated genomic DNA (~10ng/µL) and incubating the mixture at 37°C for 1 hour. A 1% agarose gel with 0.5µl of in-gel ethidium bromide stain (10mg/ml) was loaded with 0.4µl of the restriction enzyme digest and run at 80 volts for 30 minutes to verify the digest successfully cut the DNA into 500-1000 base pair fragments.

Shrimp Alkaline Phosphate (SAP) digestion was performed on each of the successful restriction enzyme digests by adding 2µL SAP (1U/µl), 0.5µl NEB Buffer #2, and 2.5µl dH2O and incubating at 37°C for 20 min, followed by 80°C for 20 min to deactivate the enzymes. This removed the phosphate from the 5' end of the DNA fragments to prevent them from self-ligating.

A mixture containing 200µl of 100uM SNX-forward (5'- CTA AAG CCT TGC TAG CAG AAG C -3'), 200µl of 100uM SNX-reverse phosphorylated at the 5' end (5'­GCT TCT GCT AGC AAG GCC TT A GAA AA -3 ') and 4µl of 5M NaCl was heated to 95°C for 5 minutes and then slowly cooled to room temperature in order to make dsSNX linkers. The linkers were added onto each end of the DNA fragments to provide a binding site for subsequent recovery PCR reactions (Figure 2) (Hamilton et al. 1999). Linker ligation was performed by combining 7.0µl dsSNX linkers (10µM), 2.5µl 10x Ligase Buffer, 1.0µl DNA ligase, 1.0µl Xmn I (NEB catalog #R0194S), and 13.5µl dH2O and incubating it overnight at 16°C. To verify linker ligation success, a 25µl PCR reaction was set up with final concentrations 20 mM Tris pH 8.4, 50 mM KCl, 2.5 µg/ml
BSA, 0.5 μM SNX primer, 2.5 mM MgCl₂, 150 μM dNTPs, 0.2 units of Taq DNA Polymerase, and 2.0μl of linker ligated fragments. Cycling parameters were 95°C for 2 min. followed by 20 cycles of 95°C for 20 sec., 60°C for 20 sec., and 72°C for 1.5 minutes. Linker ligation PCR product was run on a gel as previously described to verify that a smear of fragments ca. 500-1000 base pairs in size were amplified.

A reaction was set up on the linker ligated DNA to complete the bond between the 3’ end of the linker and the 5’ end of the genomic fragment. A PCR type reaction of 2.5μl 10xPCR buffer (200 mM Tris pH 8.4, 500 mM KCl), 1.5μl MgCl₂ (25mM), 2.5μl 10xBSA (250μg/ml), 1.5μl dNTP (10mM;1.5mM each), 11.8μl dH₂O, 5.0μl Linker ligation, and 0.2μl Taq Polymerase (~5units/μl) was combined and incubated at 72°C for 30 minutes.

Two enrichments were performed on the nick-repaired linker ligation to capture DNA fragments with microsatellite sequences complementary to the microsatellite oligo probes and wash away all other DNA fragments. The first enrichment was made using oligo mix #2 containing dinucleotide and trinucleotide probes (Table 2). The second enrichment was made using oligo mixes #4 and #6 containing tetranucleotide probes. Oligo hybridization was set up by combining 70μl hybridization buffer (6xSSC, 0.1% SDS), 5.0μl biotinylated microsatellite probe mix (10-20uM each) and 25μl of nick-repaired linker ligated DNA. The mixture was heated to 95°C for 5 minutes and slowly cooled to room temperature. To capture the probed microsatellite fragments, 300μl of TBT (0.1M Tris pH 7.5, 0.1% Tween-20) and 25μl of Avidin D beads (Vectorc Avidin D Beads Catalog #A-2020) were added and the mixture was shaken at 50°C for 1 hour. The samples were centrifuged at 15,000x for 1 minute and the supernatant was removed. The
avidin beads were washed 3 times using 400µl TBST (0.1M Tris pH=8.0, 150mM NaCl, and 0.1% Tween 20), making sure to resuspend the beads well and centrifuging them at 15,000x for 1 minute each time. Three additional washes of higher stringency were performed using 400µl Wash Solution (0.2x SSC and 0.1% SDS). The final wash was performed using 100µl TLE and an incubation at 95°C for five minutes to elute the probed microsatellite DNA containing fragments. To verify enrichment success a 25µl PCR reaction was set up with final concentrations 20 mM Tris pH 8.4, 50 mM KCl, 25.0 µg/ml BSA, 0.5 µM PigSNX-forward primer (5' - GTT TCT AAG GCC TTG CTA GCA GA -3'), 2 mM MgCl₂, 150 µM dNTPs, 0.4 unit Taq DNA Polymerase, and 5.0µl of enriched elution. Cycling parameters were 40 cycles of 95°C for 20 sec., 60°C for 20 sec., and 72°C for 1.5 minutes, and a final incubation at 72°C for 30 minutes to add a poly A-tail for TA cloning. PCR product was electrophoresed on an agarose gel as previously described to ensure it was composed of fragments concentrated between 500 and 1000 base pairs.

Enriched elution PCR product was cleaned using a PolyEthylene Glycol (PEG) solution (20% PEG, 2.5M NaCl). Equal volumes of PEG solution and PCR product were mixed and incubated at 37°C for 15 min. The mixture was centrifuged at high speed (15,000 rpm) for 15 min. at room temperature and the supernatant was discarded. Next, two washes were performed by slowly dripping 75µl of ice cold 80% ethanol down the side of the microcentrifuge tube, allowing it to sit for 1 min, and discarding the ethanol. The sample was then dried using a centrivap at low heat for several minutes until all ethanol was evaporated. The pellet was then resolubilized in 25µl TLE.
PCR product fragments were inserted into plasmids using Promega’s pGem®-T Vector System (Promega Catalog #A-3600). Specifically, 5µl of pGem-T’s 2x Rapid Ligation Buffer, 1µl pGem-T vector, 1µl of T4 ligase, and 3µl of adenlyated enriched elution PCR product were combined and incubated at 16°C overnight.

Plasmids were inserted into XL10 Gold electrocompetent bacteria cells (Strategene) to isolate the various microsatellite containing DNA fragments. The plasmid ligation mixture was combined with 20µl of TLE and heated to 65°C for 20 minutes. Next, 1µl of the ligation mix was combined with 40µl of competent cells, transferred to a 2mm cuvette, and chilled on ice for 1 minute. The cuvette was placed in an ECM® 399 electrotransporation unit (BTX, San Diego, CA) and a 2400v pulse of electricity was passed through the sample. Immediately following, 960µl of Lutria Broth (LB broth) was added to the transformed cells and mixed thoroughly. The cells were transferred to a 3ml culture tube containing 3ml of LB broth+ampicillin (Amp) (50µg/ml final conc.) and incubated at 37°C in an orbital shaker at 250 rpm for 1 hour. LB agar + Amp (50µg/ml final conc.) plates were prepared by applying and spreading 7µl of IPTG (100mg/ml) and 40µl of X-gal (20mg/ml) onto the agar surface. Plates were pre-warmed to 37°C before they were spread with 50µl of transformed bacteria. The bacteria samples were grown overnight at 37°C.

A 1.5ml deep well plate was prepared by adding 500µl of LB broth + Amp, and a thin-walled PCR plate was prepared by adding 100µl of deionized water. Colonies were then picked using sterile toothpicks and transferred both to the deionized water plate and to the corresponding well of the deep well plate containing LB broth. A PCR reaction was set up using inoculated water as template. PCR final concentrations were 10 mM
Tris pH 8.4, 50 mM KCl, 25.0 µg/ml BSA, 0.25 µM M13 forward primer, 0.25µM M13 reverse primer, 1.5 mM MgCl2, 1.5 mM dNTPs, 0.5 units Taq DNA Polymerase, and 5.0µl of bacterial colony suspended in diH2O as DNA template. Cycling parameters were an initial denaturation at 94° C for 5 minutes followed by 35 cycles of 95° C for 20 seconds, 50° C for 20 seconds, and 72° C for 1 minute 30 seconds. The bacteria-inoculated LB broth was incubated at 37°C overnight in an orbital shaker rotating at 250 rpm. LB broth + Amp containing 30% glycerol was added in 500µl volumes and the deep well plate was stored at -70°C.

PEG precipitation was performed on the colony PCR products as previously described, and cleaned product was run on a 1% agarose gel with ethidium bromide stain to quantify size and concentration. Sequencing reactions were set up in 10µl volumes using 0.33µM primer (M13 forward or reverse), big dye terminators using ABI specifications except that the terminator mix was diluted 1:1 with halfBD™ (GENPAK LTD., Stony Brook, New York), and 10ng of PCR template for 100 base pairs of desired sequence product length. Thermal cycling parameters were 40 cycles of 96°C for 10 sec, 50°C for 5 sec, and 60°C for 4 minutes. Sequence reactions were precipitated as previously described and run on an ABI 377 automated sequencer.

Sequence chromatograms were imported into Sequencher 4.1. Forward and reverse sequences were assembled, vector and SNX linkers were trimmed, sequences were edited, and the sequences were exported as concensus files. The concensus files were screened for microsatellites using Ephemeris 1.0 (program available at http://www.uga.edu/srel/DNA_Lab/index.htm). Oligo 6.6 (Molecular Biology Insights Inc.) was used to design primers for sequences that contained microsatellites. Major
criteria used to design primer pairs were as follows: primer length between 16 and 22 base pairs, product length between 100 and 400 base pairs, internal stability of upper and lower primers were lower at the 3' end than at the 5' end, hairpins unstable at 21°C or higher, and primer melting temperatures range between 55 and 60°C. To one of each primer pair a CAG (5'- CAG TCG GGC GTC ATC A -3') or M13 (5'- GGA AAC AGC TAT GAC CAT -3') tag was attached to the 5' end. This allowed labeling of PCR product by using a universal fluorescent dye-labeled M13 or CAG primer (Boutin-Ganache et al. 2001). The newly designed dollar sunfish primers were synthesized by Integrated DNA Technologies Incorporated (IDT, Inc., Coralville, IA).

Primer pairs were optimized using 8 different dollar sunfish DNA samples. PCR final concentrations for optimizing reactions were 10 mM Tris pH 8.4, 50 mM KCl, 25.0 µg/ml BSA, 0.4 µM unlabeled primer, 0.04µM tag labeled primer, 0.36µM unveral dye labeled primer, 1.5 mM MgCl₂, 1.5 mM dNTPs, 0.5 units Taq DNA Polymerase, and 50ng DNA template. M13 and CAG universal primers were labeled with a FAM or HEX fluorescent dye. Primers were tested using touchdown thermal cycling programs with initial annealing temperatures of 65°C, 60°C, or 55°C. PCR products were run on a 1% agarose gel using ethidium bromide stain. Comparisons were made to determine which touchdown protocol was optimal for each primer pair. Additional DNA samples were amplified using optimized PCR conditions to examine for polymorphism. Ultimately, polymorphism testing was attempted on 3 individuals from each population, totaling 21 individuals. PCR product was run with an internal genSIZE T350 or T500 Rox size standard (Genpak, St. James, NY) on Perkin Elmer ABI 377 Automated Sequencer using GeneScan v3.1. Chromatograms were imported into Genotyper v2.5 to determine number
of alleles per individual and allele size. Genepop 3.1c (Raymond & Rousset 1995) was used to calculate observed heterozygosity among the individuals sampled (note that individuals are from various populations).

Aging Procedure

Otoliths were removed and examined to determine age using methods similar to those described by Hales and Belk (1992). Specifically, otoliths were mounted concave side down on heated glass slides using Krystal Bond clear, transparent thermoplastic cement (Buehler Company, Lake Bluffs, Illinois, USA). The otoliths were sanded using 600 and 2000 grit sandpaper and water until rings were clearly visible. Age assessment was performed using a dissecting microscope with a dark background to count the number of yearly growth rings, or areas of condensed daily growth rings created due to decreased growth during cooler weather. Areas of reduced bone growth appear as a dark ring when light is passed through the otolith. The number of dark rings for each otolith was counted to determine fish age in years. Age determinations were made independently by the author and Dr. Robert Fischer. Conflicting age determinations were mutually re-examined and evaluated until they were resolved.

Radiocesium Analysis

Caudal peduncles were used to determine radiocesium levels (Bq/g wet mass) using a Minaxi Auto-Gamma® 5000 Series counter (Packard, Downers Grove, IL). This equipment uses a 7.62cm well-type NaI detector to measure photons in the 550-760KeV range. Each tissue sample was counted for 90 minutes to achieve a standard deviation
Background and a low activity standard with the same geometry as tissue samples were measured to determine detector efficiency.

IV. Results

Mitochondrial Control Region

Sequences were determined for 327 bp from 135 individuals. Approximately 19 individuals were sampled per population (Pond C N=18, Fire Pond N=19, Dicks Pond N=19, Wetland 41 N=20), except for Par Pond with 59 individuals (North Arm N=20, Hot Arm N=20, West Arm N=19). Figure 3 shows dollar sunfish control region sequence aligned with mud sunfish (*Acantharchus pomotis*) and largemouth bass (*Micropterus salmoides*) haplotypes. No variation was found within or between the 7 populations of dollar sunfish sampled. Base pair composition, in particular GC content of the control region examined was comparable for dollar sunfish (33.3%), mud sunfish (32.1%) and largemouth bass (35.8%). A search was performed at the National Center for Biotechnology Information (NCBI) website “http://www.ncbi.nlm.nih.gov/” to examine what types of organisms have a similar sequence in their genome. The search matched the control region segment sequenced for *L. marginatus* with Percidae family control region sequences including genera *Etheostoma, Percina, Perca, Gymnocephalus*, and *Stizostedion.*
Centrarchidae Microsatellites for Dollar Sunfish

Six of twelve microsatellite loci designed for other sunfish species amplified successfully for dollar sunfish. Three of those loci were polymorphic: Lma 120 yielded 8 alleles (N=124) with examination of individuals from all populations. The number of Lma 120 alleles for each population were 5 alleles in Par Pond, 5 alleles in Pond C, 5 alleles in Fire Pond, 2 alleles in Dicks Pond, and 2 alleles in Wetland 41. Lma 20 showed 11 alleles (N=37) with examination of individuals from Pond C, Par Pond, and Fire Pond. Rb 7 yielded 14 alleles (N=17) with examination of individuals from Pond C and Dicks Pond. Loci Lma 12 (N=23), Lma 29 (N=23), and MS 19 (N=8) were monomorphic. Finally, loci RB 20, Lma 21, Lma 116, Lma 121, Lma 124, and MS 21 had high amounts of nonspecific amplification and were not scorable. Because only three previously designed primers may be used for dollar sunfish, more microsatellite loci are needed to adequately compare genetic structure of different populations.

Microsatellite Development Results

Nine microsatellite primer pairs were successfully developed for dollar sunfish (Table 3). These were tested on 2-3 individuals from each sample location so that N=20. The dollar sunfish microsatellite primers developed yielded a high number of alleles ranging from 4-14 (mean = 8.6), and observed heterozygosities ranged from 0.500 to 0.857 (mean heterozygosity = 0.6337). Along with Rb 7, Lma 20 and Lma 120, these new microsatellite primers allow for adequate genetic comparisons of dollar sunfish populations.
Aging and Radiocesium 137 Results

Age structure comparisons were performed on dollar sunfish from various sample locations (N=243) (Table 4). Par North, Par Hot, Fire Pond, Dicks Pond, and Wetland 41 individuals sampled create an age class trend that rises to a peak at 2.5 years and then declines (Figure 4). Par West and Pond C sample sites varied from that trend by peaking at 1.5 years of age with a less rapid decline in numbers of fish older than 1.5 years. When all locations were combined, the overall age class structure showed the most frequently sampled age for dollar sunfish was 2.5 (Figure 5).

Radiocesium analysis was performed on dollar sunfish (N=243) from the seven sample locations previously described. All samples from Fire Pond, Dicks Pond, Wetland 41, and the North and Hot Arms of Par Pond were below the detection limit. Detectable amounts of $^{137}$Cs were found in individuals from Pond C (mean = 2.34 bq/g, N=5) and the West Arm of Par (mean = 1.57 bq/g, N=9). However, samples testing positive were barely above the detection limit (calculated for each sample according to mass), rendering inadequate data to make comparisons between dollar sunfish age and $^{137}$Cs concentration.

V. Discussion

The absence of sequence variation found in the dollar sunfish control region was unanticipated. Billington & Herbert (1991) describe a plethora of mitochondrial variation among many different fishes. Mitochondrial size variation was found in white sturgeon (Acipenser transmontus), striped bass (Morone saxatilis), white perch (Morone Americana), and in American shad (Alosa sapidissima) (Billington & Hebert 1991).
Investigations by many researchers have revealed that all length variants found were located in the mitochondrial control region. Further studies found bluegill (*L. macrochirus*), spotted sunfish (*L. punctatus*), redear sunfish (*L. microlophus*), and warmouth (*L. gulosus*) exhibit intraspecific sequence divergence of their mitochondrial DNA (Billington & Hebert 1991). A study performed on largemouth bass at the SRS showed high amounts of mtDNA control region variation in fish sampled from both experimental and reference ponds (T. Glenn unpublished). The findings of these and other previous fish mtDNA studies, in particular those involving control region variation, demonstrate why sequence variation was expected in the dollar sunfish control region.

There are several possible scenarios that would cause little or no variation to occur in dollar sunfish control region sequences. It is possible that a bottleneck event occurred for dollar sunfish at the Savannah River Site causing fixation of mitochondrial DNA D-loop sequence. However, this is an unlikely scenario because reference populations showed no variation, high mutation rate for mitochondrial DNA control region and conditions in contaminated ponds encourage point mutations. In addition, reference sites used in this study are in different watersheds than Ponds C and Par Pond (Staton *et al.* 2002). These factors suggest that a bottleneck event is not the reason for finding no mtDNA control region sequence variation in SRS dollar sunfish.

Another possibility is that a phenomenon called nuclear introgression has occurred in which a copy of the mtDNA control region has been translocated into the nucleus, so there are two copies of the same gene found in the organism (Quinn 1997). A nuclear copy of a mtDNA gene is referred to as a “numt”. Insertion instances of mtDNA sequences into the nuclear genome have been described by Smith *et al.* (1992) for
akodontine rodents and by Lopez *et al.* (1994) for domestic cats. It is possible that use of a whole genomic DNA extraction and a PCR reaction that was possibly biased for preferential amplification of the nuclear copy of this gene might result in sequences yielding only the nuclear copy of this gene. It is more likely for a nuclear gene to have no variation because of a recent introgression event and the ability of nuclear DNA polymerase to correct errors made during replication. Despite complications caused by the presence of a gene in multiple locations and uncertainty for which copy is being observed, an opportunity is created allowing for examination of mutation rate differences between single-copy nuclear DNA and mtDNA. Arctander (1995) used a nuclear copy of the mitochondrial cytochrome b gene in *Scytalopus spp.* to show that particular mtDNA gene was evolving 13.6 times faster than its nuclear copy. Zischler *et al.* (1995) were able to use a different application to observe a similar phenomenon. They utilized a human pseudogene as an outgroup to aid in examining the origin of modern humans. These studies demonstrate the opportunities provided by nuclear mitochondrial sequences. A unique situation is revealed for future ecotoxicological research if dollar sunfish living at the SRS do indeed have a nuclear copy of the mitochondrial control region.

Future studies should include isolation of mtDNA using mitochondrial rich tissues such as liver or muscle. Following procedures used in this study, mtDNA extract should be amplified using primers L15998-Pro and LMB-H396, sequenced and compared to sequences obtained using whole genomic DNA extract. If there is evidence to support the possibility that *L. marginatus* at the SRS have a “numt” gene, then studies of mitochondrial and nuclear evolutionary rate studies could be pursued. Regardless of the
potential avenues of research that mitochondrial control region examination have uncovered, results indicate a different molecular technique will be more beneficial for examining genetic variation of dollar sunfish in the contaminated and uncontaminated environments at the SRS. The use of single copy nuclear DNA does not restrict genetic analysis to a haploid mode of inheritance and high amounts of variation are often found for non-coding DNA such as microsatellites.

Examination of microsatellite loci designed for largemouth bass, bluegill and redbreast sunfish found that several of those primers amplified using dollar sunfish DNA and three of them, Lma 120, Lma 20, and Rb 7 were polymorphic. Success of these primers indicates they are likely to work on other closely related species. Inclusion of additional optimization strategies for primers showing non-specific banding patterns may allow them to provide useful data for dollar sunfish and other related species. Evidence obtained from locus L120 supports suspicion that control ponds were chosen poorly with respect to size. Lma 120 showed higher amounts of variation in Ponds C, Par Pond and Fire Pond than in Dicks Pond and Wetland 41. Reference populations (Fire Pond, Dicks Pond, and Wetland 41) were all approximately 0.01 km², whereas experimental populations were approximately 0.5 km² or larger. In future studies, particular attention to population size and pond size need to be taken into account with reference selection.

Dollar sunfish are ideal subjects for many future studies, especially paternity, because of their small size and shallow nesting location. As mentioned earlier, dollar sunfish are less likely to have genetic patterns altered as a result of stocking, making them more desirable for use as ecological indicators. Three microsatellite primers did not provide adequate amounts of information to infer conclusions regarding genetic variation.
Additional primers were mandatory for the inclusion of this species in molecular studies. The addition of primers to amplify 9 polymorphic microsatellite loci from *L. marginatus* will benefit researchers seeking to pursue studies of dollar sunfish, and potentially for research involving other closely related species.

Aging data showed that the method of sampling used was biased toward capturing larger individuals (>1 year old). Species identification is often more difficult for young individuals, so young fish were typically not kept for analysis due to species uncertainty. In the absence of age bias created by sampling method, species uncertainty and unusual circumstances involving environmental conditions, examination of dollar sunfish population composition should show that the vast majority of fish in the population are ≤1 year old and there is a decrease in individuals composing each subsequent age class.

Radiocesium measurements have provided very little useful data at the present time. Fish samples were collected during the month of August (1999). According to Peles *et al.* (2000), despite higher water $^{137}\text{Cs}$ concentrations during warmer times of the year, one should expect the quantity of radiocesium in muscle tissue to be at its lowest concentration during the late summer and fall because fish are able to eliminate $^{137}\text{Cs}$ with increased metabolism. The use of small amounts of tissue in combination with long count time led to a high standard deviation on the particular gamma counter that was used in this study. Late summer sampling coupled with small tissue samples and limited capabilities of the gamma counting machine (which rounded counts per minute and then averages the resulting values) has postponed obtaining usable data thus far. When $^{137}\text{Cs}$ counts are repeated, the entire remaining fish sample will be measured rather than caudal peduncles and a different gamma radiation counter will be used that will not introduce
large amounts of rounding error. The dollar sunfish’s ability to excrete $^{137}\text{Cs}$, along with its small size, short life, and low level on the food chain may cause this species to be less than useful for examining radiocesium in an ecosystem than first anticipated. However, further investigations may find otherwise if fish are sampled at various times throughout the year and larger amounts of tissue are used for radiocesium detection.

Overall, dollar sunfish show great potential for use in a variety of future studies involving mitochondrial DNA, including tests to see if there are two copies of the control region for dollar sunfish. If a numt is discovered, then comparison of gene sequence and studies of mutation rate differences for nuclear and mitochondrial DNA should be pursued. The availability of microsatellite primers provides research possibilities in areas such as relatedness, parentage and alternate mating strategies, population dynamics, and comparisons among species. Hopefully, further investigations of radiocesium bioaccumulation in dollar sunfish muscle tissues will show that this species can successfully be used as an ecological indicator.
Literature Cited


Table 1. Centrarchidae microsatellite primers tested using Lepomis marginatus DNA. Number of alleles and size ranges provided for primer pairs that successfully amplified for L. marginatus. N = Number of Individuals Tested, N/A = not applicable, ND = not determined

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primer Sequence 5' ---&gt; 3'</th>
<th>Dye Label</th>
<th>Touchdown Thermalcycling Program</th>
<th>N</th>
<th>Number of Alleles</th>
<th>Size (bp)</th>
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<td>TD 65</td>
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<td>Lma 124</td>
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<td>MS 19</td>
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Table 2. Oligo mixes used to create an enriched library for *Lepomis marginatus*.

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<th>Oligo</th>
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<th>Concentration (µM)</th>
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</tr>
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<tr>
<td>(AACT)8</td>
<td>14 14 14 14 14 14</td>
<td>200</td>
<td>100 200 100 200 100 200 100 200 100 200</td>
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</table>

Mix 2: (TG)12, (AG)12, (AAG)8, (ATC)8, (AAC)8, (AAT)12, (ACT)12, (AAAT)8, (AACT)8

Mix 4: (AAGT)8, (ACAT)8, (AGAT)8, (AACC)5, (AACG)5

Mix 6: (AAGC)5, (AAGG)5, (ATCC)5
Table 3. Characterization of nine primer pairs that amplify microsatellites from Lepomis marginatus. Sequences used to introduce sites for universal fluorescent primers are in italics and a smaller font. Touchdown Temperature refers to the initial annealing temperature of the Touchdown protocol used. *N* refers to sample size. Size Range refers to the observed distribution of alleles at each locus. Clone Size refers to the size of the PCR product amplified from the clone used to develop each locus which was identical to the size predicted. ND = not determined.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primer Sequence 5' ---&gt; 3'</th>
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<th>Touchdown Temperature</th>
<th>Repeat Sequence</th>
<th>N</th>
<th>Number of Alleles</th>
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<tr>
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Table 4. *Lepomis marginatus* age data.

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<tr>
<th>Sample Site</th>
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<th>Mean Age</th>
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<th>Mode</th>
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<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Dicks Pond</td>
<td>29</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Wetland 41</td>
<td>30</td>
<td>2.7</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Cummulative</td>
<td>223</td>
<td>2.4</td>
<td>2.5</td>
<td>2.5</td>
</tr>
</tbody>
</table>
Figure Legend

Figure 1. The Savannah River Site including sample locations at the three arms of Par Pond, Pond C, Fire Pond, Dicks Pond, and Wetland 41.

Figure 2. Super SNX Linkers attempting to ligate to each other. Shown are the restriction enzyme site XmnI for prevention of linker dimerization, restriction enzyme sites Stu I and Nhe I for sticky ended ligation into a plasmid, and a poly-A tail to ensure the correct end of the linker is ligated to DNA fragments.

Figure 3. Dollar sunfish control region sequence aligned with mud sunfish (Acantharchus pomotis) and largemouth bass (Micropterus salmoides) control region sequences.

Figure 4. Dollar sunfish age class trends.

Figure 5. Savannah River Site dollar sunfish age composition.
Figure 1
Figure 2

5' - CTAAGGCCTTGCTAGCAGAAGC
- pGCTTCTGCTAGCAAGGCCTTAGAAAA -3'
3' - AAAAGATTCCGGAACGATCGTCTTCGp-
CGAAGACGATCGTTCCGGAATC -5'

A-tail  Stu I  Xmn I  Nhe I

A-tail  Stu I  A-tail
Figure 4

Dollar Sunfish Population Age Structure

- Par North
- Par West
- Par Hot
- Pond C
- Fire Pond
- Dicks Pond
- Wetland 41

Frequency (# Fish)

Age (years)
Figure 5

SRS Dollar Sunfish Age Composition

Percentage of Fish

0% 5% 10% 15% 20% 25% 30% 35%

Age (years)

All Fish Sampled