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Genetic Assessment of Rare Blackbanded Sunfish (Enneacanthus Chaetodon) Populations in Virginia

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GENETIC ASSESSMENT OF RARE BLACKBANDED SUNFISH (*ENNEACANTHUS CHAETODON*) POPULATIONS IN VIRGINIA

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biology at Virginia Commonwealth University

By

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Abstract

Genetic assessment of rare blackbanded sunfish (*Enneacanthus chaetodon*) populations in Virginia

By Diana Marie Kercher, B.S.

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Major Director: Bonnie L. Brown
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Enneacanthus chaetodon, the blackbanded sunfish, has become increasingly rare throughout its distribution in the Eastern United States. In Virginia, *E. chaetodon* maintains an endangered status and individuals persist in six populations. Mitochondrial DNA (mtDNA) and microsatellite data were assessed to determine the genetic characters and gene diversity of the Virginia populations. The results of these analyses were then compared to five additional populations; four from New Jersey and one from North Carolina that were known to have relatively good fitness and were not impacted severely by habitat alteration. The results of this study are relevant to selection of proper management techniques and strategies for this species. Mitochondrial DNA analyses

detected no variation in the Virginia populations but significant ($P < 0.05$) variation in the other sampled groups. Investigation of these eleven populations with the use of microsatellite markers uncovered low levels of genetic diversity (heterozygosity < 0.5) in Virginia populations and extremely high levels ($F > 0.2$) of inbreeding. The New Jersey and North Carolina populations demonstrated lower amounts of inbreeding than populations in Virginia. New Jersey displayed a significant ($P < 0.05$) amount of subdivision among populations compared to Virginia. Hypothesis testing supported the contention that the regions are significantly different from one another and that Virginia populations may have gone through one or more population bottlenecks in the past, explaining the low levels of diversity observed and significantly high inbreeding coefficients. Captive breeding programs could be implemented as a management measure to increase population numbers and restore fish into areas where they have been known to inhabit in the recent past. From a proper management perspective, habitat protection and maintenance are more important than supplementation to population survival. Success of either approach with Virginia populations would provide a useful model for managing small populations of blackbanded sunfish in other regions.

VCU Introduction

Small ecological populations are subject to various natural processes that act upon their genetic makeup due to their limited sizes. These processes can be separated into two categories: random processes, such as genetic drift, and the effects of inbreeding (Franklin 1980). More research to date has been devoted to applying classical population genetics to issues of population management since the realization that intervention is necessary to prevent the extinction of multitudes of species. Managing populations with small numbers (< 50) of individuals is a difficult task because they are more susceptible to changes inflicted upon them than larger populations due to decreased genetic variability and consequent reduction in fitness. Measures of fitness can be considered in two different ways, by the individual and the population. On the individual level, reduced heterozygosity and higher levels of inbreeding are correlated with reductions in sperm production, fecundity, and sterility (O'Brien 1994). However, fitness also relates to long-term concerns regarding loss of variability and the ability of a population to maintain itself (Franklin 1980). Such long term fitness is related directly to adaptive potential, which influences a species' adaptability to environmental changes, maintenance of evolutionary fitness, and overall population homeostasis (Soulé 1987).

Genetic variation is determined primarily by the joint actions of natural selection, genetic drift, mutation, and migration. In small, reproductively isolated populations,

rapid changes in allele frequencies can occur from one generation to the next, independent of mutation, recombination, or natural selection. These allele changes are due solely to stochastic factors such as genetic drift (Wright 1946). The smaller the population, the more susceptible it is to random changes. Any allele, deleterious, beneficial, or neutral is more likely to be lost from a small population's gene pool than from a large one (Hartl 2000). In small populations, the relative importance of genetic drift can be observed in the expression of deleterious alleles that can become more frequent and, in extreme cases, fixed in a population (Hartl 2000). Drift is predicted to have the greatest effect when the number of breeding adults is restricted, if even only for short periods of time (Slatkin 1987).

Population bottlenecks can influence and increase the effects of genetic drift. Bottlenecks for fish populations result from environmental changes such as altered hydrology or weather conditions, or from anthropogenic actions such as damming, habitat alteration or overharvesting. If as a result of such an event, a significant proportion of a population (more than 50%) is prevented from reproducing, the survivors of a population bottleneck are not generally representative of the original population. Populations that have experienced a recent reduction of their effective population size exhibit a correlated reduction in the numbers of alleles and gene diversity at polymorphic loci; allele numbers declining faster than gene diversity is reduced. The rate of genetic drift in recently bottlenecked populations has been observed to be inversely proportional to the augmented population size (Luikart et al. 1998). Thus, in a bottlenecked (or any small population), the observed gene diversity is likely to be higher than the expected

equilibrium gene diversity (Luikart et al. 1998). Such a scenario can be distinguished from an unimpacted population at mutation-drift equilibrium (i.e., its effective number of breeders has remained fairly constant over the recent past), where there is approximately an equal chance of a locus showing a gene diversity excess or a gene diversity deficit.

Inbreeding often occurs in small populations. The degree of these effects depends on the genetic makeup of the species and on how genotypes interact with the environment (Vucetich & Waite 2001). Offspring of related parents have a far higher number of homozygous loci than offspring of unrelated parents (Hartl 2000). When many breeding pairs in a population are related, rare alleles can be lost and the frequency of deleterious alleles can increase or, in extreme cases, become fixed. As a result of inbreeding, the overall genetic variability of a population is reduced. Inbreeding depression, a typical outcome of such a scenario, reduces the fitness of a species. Another extreme case of inbreeding depression would be the accumulation of a multitude of deleterious mutations in a small population. Most organisms carry numerous deleterious alleles, but the population effects of these are masked because many individuals in a population are in a heterozygous state. In genetically diverse populations, the chance of both parents giving a deleterious allele of the same gene to their offspring is minimal (Hedrick & Kalinowski 2000). However, as a result of the cumulative effect of genetic drift in small populations, offspring may receive deleterious alleles of a gene from both parents. All individuals may suffer from inbreeding depression and experience decreased individual fitness leading to eventual failure of the population to adapt and survive.

Enneacanthus chaetodon, the blackbanded sunfish, has a distribution ranging

from New Jersey to central Florida along the North American Atlantic and Gulf slopes (Lee et al. 1980). E. chaetodon was placed on the Virginia state endangered species list in 1987, when the species was found to occur only in the Nottoway and Blackwater River watersheds of the Chowan River System which encompasses 5,900 km^2 and whose tributaries include the Nottoway River, the Meherrin River, and the Blackwater River. Blackbanded sunfish inhabit four specific areas in the Chowan Drainage: Cypress Swamp (Surry County), Blackwater Swamp (Prince George County), Harrell's Pond (impoundment of the Coppahaunk Swamp, Sussex County) and Game Refuge Lake (impoundment of Dobie Swamp, Sussex). The former three areas are all tributaries to the Blackwater River; the latter a tributary of the Nottoway River. Only one specimen has been collected from the Cypress Swamp (1985), and its presence has not been detected in Blackwater Swamp since 1973 (Terwilliger 1991). Since 1996, six populations have been found in the Blackwater Drainage of Virginia. These six populations were named for their location: Harrell's Millpond, Lake Binford, Cupp Pond (private pond), "602" (small pond off route 602), Game Refuge Lake, and Neblett's Millpond. Harrell's Pond and Lake Binford are not connected, whereas the others are hypothesized to experience one way gene flow. Cupp Pond and "602" are both parts of the Dobie Swamp above Game Refuge Lake. Many farms border the rivers in this system and no blackbanded sunfish populations collected downstream of farms with swine or dense row crops. All samples in this study were collected upstream, where there was limited farming activity.

The blackbanded sunfish is a member of the Class *Actinopterygii*, Order *Perciformes*, and Family *Centrarchidae*. Other members of this genus include *E.*

gloriosus, the bluespotted sunfish, and *E. obesus*, the banded sunfish. The blackbanded sunfish is distinguished from the latter two species by bold black bars running vertically along the length of its small, laterally compressed body and mottled fins (Jenkins et al. 1975). Blackbanded sunfish by virtue of their coloration, small size, rounded caudal fin, and tolerance to acidic water conditions are well-suited to vegetated ponds, quiet sand and mud-bottomed pools, backwaters of creeks, and small to medium rivers (Schwartz 1961). Virginia's coastal plain streams have a varied hydrology depending on the time of year. Channels and streams that may be dry in mid summer are often flooded by thunderstorms or hurricanes in the spring and fall. Beaver damming also contributes to habitat alteration by creating ponds in which dense aquatic vegetation persists, creating a favorable habitat in which blackbanded sunfish can feed, spawn, and hide. Adult specimens have been recorded to reach lengths of 30-60 mm, and live a maximum of three to four years (Jenkins & Burkhead 1994). In Maryland, individuals lived 3-4 years, whereas in Delaware most females lived 2 years and males only 1 year. In Virginia both sexes were recorded to survive to age 3 (Jenkins and Burkhead 1994). Spawning has been observed as early as March in North Carolina and gravid females were observed in Delaware from early May to late June at water temperatures of 21-28°C (Burkhead & Jenkins 1991). Blackbanded sunfish consume various zooplankton, aquatic insects (e.g., midge larvae), and crustaceans associated with aquatic macrophytes (Cooper 1983).

Potential anthropogenic threats to the blackbanded sunfish include destruction of habitat by drainage of lowlands, chemical, mechanical, and biological removal of aquatic vegetation, direct and indirect effects of chemical pollution (e.g. agricultural runoff), poor

logging and forest-clearing practices and introduction of non-native fishes (Jenkins & Burkhead 1993). An additional threatening factor for blackbanded sunfish and its habitat in Virginia is potential location of a coal-fired power plant along the Blackwater River System, approximately 1.5 km from the Blackwater Drainage area (R. Sobin www.deq.virginia.gov/air/sab/powpltfs.pdf).

Management options to facilitate blackbanded sunfish recovery include: maintaining habitat quality without interfering with *E. chaetodon* populations, conducting captive breeding to supplement existing *E. chaetodon* in existing populations, and reintroducing blackbanded sunfish to known historical habitats where they are no longer found (Smoger et al. 1999). Assuming habitat integrity is adequately addressed, supplementation and reintroduction are viable prospects. However, a finding of high levels of genetic variation coupled with significant divergence among the Virginia populations, relative to divergence observed among the populations in Virginia and other states, would indicate that the most prudent recovery strategy would be to maintain separate supplementation programs for each population.

Genetic assessment of rare blackbanded sunfish (*Enneacanthus chaetodon*) populations in Virginia

Running title: Genetic diversity of endangered Virginia sunfish

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Abstract

Enneacanthus chaetodon, the blackbanded sunfish, has become increasingly rare throughout its distribution in North America. In Virginia, *E. chaetodon* maintains an endangered status and individuals persist in only six populations. Mitochondrial DNA (mtDNA) and microsatellites were assessed to determine population characteristics and genetic diversity of the Virginia populations. These data were compared to five additional populations: four from New Jersey and one from North Carolina. MtDNA detected no variation in the Virginia populations but substantial levels in the other populations. Microsatellites exhibited lower levels of genetic diversity and higher levels of inbreeding in Virginia populations than those sampled in New Jersey and North Carolina. New Jersey displayed a significant amount of subdivision among populations compared to Virginia. Hypothesis testing supported the contention that the regions are significantly different from one another and that Virginia populations have gone through one or more population bottlenecks in the recent past, accounting for the low levels of diversity observed and significantly high inbreeding coefficients. From a management perspective, habitat protection and maintenance are most vital to population survival. Captive breeding programs, if undertaken, should utilize breeders selected from the most closely related healthy populations to increase population numbers and restore fish into areas they have been known to inhabit in the recent past.

Introduction

Enneacanthus chaetodon, blackbanded sunfish, has a distribution ranging from New Jersey to central Florida along the North American Atlantic and Gulf slopes (Lee *et al.* 1980). In Virginia, they have been considered an endangered species since 1987, when surveys found them to occur only in the Nottoway and Blackwater River Watersheds of the Chowan River Drainage System. This watershed encompasses 5,900 km^2 including the Nottoway River, the Meherrin River, and the Blackwater River. Prior to the current study, blackbanded sunfish were known to inhabit four specific areas in the Chowan Drainage: Cypress Swamp (Surry County), Blackwater Swamp (Prince George County), Harrell's Pond (an impoundment of the Coppahaunk Swamp, Sussex County) and Game Refuge Lake (an impoundment of Dobie Swamp, Sussex County). The former three reservoirs are adjacent to the Blackwater River; the latter is adjacent to the Nottoway River. Only one specimen has been collected from Cypress Swamp (1985), and presence of this species has not been detected in Blackwater Swamp since 1973 (Terwilliger 1991). Since 1996, Virginia range of the species has dwindled to six populations in the Blackwater Drainage. Numerous farms border rivers in this watershed and no blackbanded sunfish populations have been detected downstream of farms with swine or dense row crops (Smith *et al.* 2000). Potential anthropogenic threats to the blackbanded sunfish include: destruction of habitat by drainage of lowlands; chemical, mechanical, and biological removal of aquatic vegetation; direct and indirect effects of chemical pollution (e.g. agricultural runoff); inappropriate logging and forest-clearing practices; and effects of non-native fishes (Jenkins & Burkhead 1993). A coal-fired

power plant proposed to be situated along the Blackwater River System may also threaten blackbanded sunfish due to additional habitat effects (Sobin 2004).

Management options to facilitate blackbanded sunfish recovery include: maintaining habitat quality without interfering with *E. chaetodon* populations, conducting captive breeding to supplement existing *E. chaetodon* in existing populations, and reintroducing blackbanded sunfish to known historical habitats where they are no longer found (Smoger 1999). Assuming habitat integrity is adequately addressed, supplementation and reintroduction are viable prospects. If relict blackbanded sunfish populations in Virginia are not significantly divergent from one another and have low levels of genetic diversity, perhaps supplementation from other known blackbanded sunfish populations (e.g., populations in nearby states) could postpone or alleviate their decline. However, a finding of high levels of genetic variation coupled with significant divergence among populations would indicate that the most prudent recovery strategy would be to maintain separate supplementation programs for each population.

Due to habitat loss and other accumulated anthropogenic disturbances over time, blackbanded sunfish have become highly fragmented throughout their range. However, the lack of prior population genetic knowledge for this species poses difficulties for management. Our approach for determining the best management strategies for blackbanded sunfish included using molecular genetic markers (mtDNA and microsatellites) to quantify genetic diversity within and among populations sampled in Virginia, New Jersey, and North Carolina. The following hypotheses were tested: Virginia populations of *E. chaetodon* are not significantly divergent from northern (NJ)

and southern (NC) populations, Virginia populations of *E. chaetodon* are not significantly divergent from one another relative to divergence observed among Virginia and other populations, and Virginia populations have not experienced population bottlenecks in the recent past.

Methods

Population sampling and DNA extraction

Surveys of Virginia *E. chaetodon* populations and specimen collections for this project were made by Virginia Department of Game and Inland Fisheries (VDGIF). More than 400 blackbanded sunfish tissue samples (fin clips) were collected during the summer and fall of 1999 from six sites in Virginia: Harrell's Millpond (36.7758 N, 77.3922 W); Lake Binford (37.1500 N, 77.2083 W); Cupp Pond, a private pond on the south branch of Dobie Swamp above Game Refuge Lake (37.0402 N, 77.2643 W); "602," referring to the pond off Sussex County Road 602 on Dobie Swamp above Game Refuge Lake (37.0443 N, 77.2513 W); Game Refuge Lake (37.0420 N, 77.2415 W); and Neblett's Millpond, on Dobie Swamp below Game Refuge Lake (37.0014 N, 77.2186 W) (Figure 1). Samples were collected from each population during July through October. Additional outgroup samples were collected in early October from four native *E. chaetodon* populations in New Jersey: Chatsworth Lake (39.8156 N, 74.5456 W), Oswego Lake (39.7264 N, 74.4928W), Presidential Lake (39.9103 N, 74.5783 W), and Horicon Lake (40.0092 N, 74.3192 W). Individuals were collected from two sampling sites in North Carolina, Beaver Dam (34.6597 N, 78.5342 W) and Gum Swamp Lake (34.4833 N, 78.7656 W)

for further comparison. Depending on the physicochemical conditions at each site, fish were captured using a variety of methods that included minnow traps, dip nets, seines, and electrofishing as described by Smith *et al.* (2000). In most cases, tissue samples consisted of non-lethal fin clips. DNA was isolated from fin clip samples using a modified STE extraction procedure (Hillis *et al.* 1996). Extracted DNA consisted largely of fragments >10,000 bp and was quantified by spectrophotometry.

Mitochondrial DNA amplification and restriction fragment analysis

Each fish was examined for its mtDNA haplotype as an initial measure of genetic biodiversity. To conduct restriction fragment analysis, three large regions collectively covering the entire mitochondrial genome were amplified. To accomplish this, we sequenced portions of the ATPase 6 and 8, cytochrome b, and 16S rRNA genes and developed primers specific for sunfish as listed in Table 1. The smallest fragment (approx. 4.5 kb and containing the d-loop) was amplified in reactions that contained 500 mM KCl, 100 mM Tris-HCl pH 9, 10% Triton X-100, 1.5 mM $MgCl_2$, 200 μM each dNTP, 15 pmol of each primer, 0.75 units of display *Taq* FL DNA polymerase (Display Systems Biotech) and 1 μl of purified DNA as template. The two slightly larger fragments required use of Elongase™ Enzyme Mix (Life Technologies). Long PCR reactions contained 60 mM Tris-SO_4 (pH 9.1), 18 mM $(NH_4)_2SO_4$, 1.8 mM $MgSO_4$, 200 μM each dNTP, 1.5 pmol each primer, 0.33 μl Elongase enzyme mix and 1 μl of template. All reactions were brought to a total volume of 15 μl with sterile deionized water. Amplifications were carried out in a PTC-100 (MJ Research, Waltham,

Massachusetts) thermal cycler and denatured for 3 min at 94°C, followed by 32 cycles of 94°C for 30 sec and a combined annealing/extension step at 69°C for 13 min. Samples were held at 4°C until analyzed by restriction digestion.

Restriction enzyme assays were performed for 15 enzymes, each in a volume of 10 µl containing 2 µl PCR product, 0.2 units restriction enzyme, 1 µl buffer supplied by the manufacturer, 1 µl BSA (1 mg/ml). Reactions were held at 37°C for 2 hr, and then stopped by the addition of 1.5 µl of loading buffer (25% glycerol, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol). Approximately 5 µl of each reaction was electrophoresed through a 1.6% agarose gel at 12 mA for 16 hr with at least two lanes of 1 kb DNA size-standard ladder, stained for 5 min in a 1:20,000 solution of ethidium bromide in 1 X TAE, and visualized and photographed under UV light with an Eagle Eye system (Stratagene). Observed fragment patterns were assigned alphabetic characters and the characters for each restriction enzyme pattern for an individual were concatenated to form the individual's haplotype. Individuals missing any portion of the restriction fragment data were excluded from statistical analysis. The statistical software package REAP (McEklroy *et al.* 1991) was used to calculate estimates of nucleotide diversity (Π) and haplotype diversity (h) within populations (Nei 1987), and nucleotide divergence (d_A) among populations (Nei & Tajima 1981.

Microsatellite DNA amplification and analysis

Microsatellites were isolated from a pool of blackbanded sunfish DNA by the methods described by Brown *et al.* (2000). More than 30 clones containing tri- and tetra-

nucleotide repeats were evaluated for Mendelian inheritance and polymorphism. Of these, five repeat loci were selected (Ech9, Ech12, Ech14, Ech32, and Ech33; Table 1) along with one locus previously published for the redbreasted sunfish (RB20) to survey nuclear DNA variation. For amplification of microsatellite loci, 0.5 µl of 1:10 diluted sunfish DNA, 0.16 µl 4mM spermidine, 0.3 µl BSA (at 0.5mg/mL), 0.5 µl each primer, 3 µl JumpStart Taq ReadyMix™, and 1.54 µl distilled water were combined in a 10 µl reaction. Amplifications were carried out in a MJ Research PTC-100 thermal cycler using two different programs, Touch 1 and Touch 2, the latter being used only for loci Ech12 and Ech14. Both programs began with a 15 min hot start at 95°C and all programs ended at 4°C until terminated. Amplification cycles for Touch 1 were 10 touchdown cycles of 94°C for 30 sec, 65°C for 30 sec (reducing 1°C/cycle) and 72°C for 30 sec, 25 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec. Amplification cycles for Touch 2 were 10 touchdown cycles of 94°C for 30 sec, 65°C for 30 sec (reducing 2°C/cycle) and 72°C for 30 sec 25 cycles of 94°C for 30 sec, 45°C for 30 sec, and 72°C for 30 sec.

Two methods were used to determine allele size. A small portion of the samples was resolved using native polyacrylmide gel electrophoresis ranging from 5-9 % stained with SYBRGreen I® nucleic acid stain (Molecular Probes, Eugene, Oregon USA). Amplification products separated in this manner were visualized and photographed under ultraviolet transillumination (Eagle Eye Stratagene). Scoring was done by hand and in accordance with previously scored gels. The majority of samples were scored on an ABI-1377 Automatic Sequencer with internal lane molecular weight standards. Data

analysis for fluorescent genotyping was performed by Lark Technologies using Genescan 3.0 software and the alleles determined using Genotyper 2.1 software (Applied Biosystems). The chromatogram for each individual reaction was checked for scoring precision and consistency between the two types of analyses was confirmed.

A descriptive baseline was created to determine the genetic diversity and variation present in each population. Statistical testing was done for all populations except 602 and Neblett's Mill Pond (due to insufficient sample sizes). Heterozygosity (observed H_o and Hardy-Weinberg expected H_e) of the microsatellite loci for each population was estimated using the population genetic software GENO (Dyer 2005). Tests for heterozygote deficit or excess were conducted by Wilcoxin sign-rank test. This program also calculated genetic differentiation among all populations and pairs of populations using Φ_{ST} statistics, according to Excoffier *et al.* (1992). Analysis of Molecular Variance (AMOVA) was performed using GENO. Exact tests for Hardy-Weinberg Equilibrium, allele frequencies, population differentiation, and effective numbers of migrants were all calculated using the GENEPOP software (Version 3.4; Raymond & Rousset 1995). Deviation from Hardy-Weinberg Equilibrium expectations used the Markov chain method and consisted of 1000 dememorization steps, 100 batches, and 1000 iterations. Pairwise genic differentiation among populations was calculated using exact tests for each locus. To evaluate potential geneflow, effective migration rate, N_em, was computed in GENEPOP using private allele frequencies and by the standard relationship of N_em to F_{ST}.

The estimated distributions of inbreeding coefficients for populations in the three regions were calculated in GENO using the adult genotypes option. These coefficient values were plotted and significance was tested ($P < 0.05$) using one-way ANOVA and Tukey's post hoc multiple comparison test to determine homogenous subsets. Isolation by distance was determined using geographic (derived from longitude/latitude measurements) and river distances (derived from a map of waterway distances) between each pair of populations. Physical distance measures were plotted against genetic distances generated by GENO to assess relationships using Mantel tests.). The MICROSAT program (Minch 1997) was used to define Nei's genetic distance (D_S) which was in turn used to construct a bootstrapped neighbor-joining tree using PHYLIP (Felsenstein 1995).

The possibility of recent effective population size reductions was examined using BOTTLENECK (Ver 1.2; Cornuet & Luikart 1996). The Wilcoxon sign-rank statistic was employed to test for heterozygosity deficiency or excess, and the allele frequency distribution mode shift analyses (Luikart *et al.* 1998) were performed using the heterozygosity data to detect the probability of recent population bottlenecks under the two-phased model (TPM) using the default settings for TPM and proportions of stepwise mutation and infinite alleles evolutionary models. The TPM was selected because it performs adequately with lower numbers of loci (as few as four loci) and smaller sample sizes than the other two models implemented by BOTTLENECK (Luikart *et al.* 1998).

Results

Population sampling

Over the period of study, 63 collections were made from 114 locations in Virginia yielding tissue from 212 individuals. On each occasion, significant effort was expended to capture as large a portion of the extant population as possible. Subsequent trips yielded no additional fish that had not previously been fin-clipped. Due to the intensity of sampling and the fact that all recaptures were fish that had previously been sampled, the Virginia samples are considered an adequate representation of the remnant blackbanded sunfish populations found at each site. Specimens were found to favor areas with dense submerged aquatic vegetation, though many areas with such suitable habitat were also found to have no blackbanded sunfish inhabitants. A total of 356 blackbanded sunfish tissue samples were included in the final analyses. Due to small population size (less than three individuals), samples from populations 602 and Neblett's Millpond were analyzed for mtDNA and microsatellites but were not included in the subsequent statistical analyses.

Mitochondrial DNA variability and phylogeny

None of the fifteen restriction endonucleases recognized cut sites in the d-loop amplicon of blackbanded sunfish and only three, *Dra* I, *Eco*RV, and *Hind*III, cleaved the other two amplified mtDNA segments. Overall, the assay encompassed approximately 0.3% of the 17,000 bp mtDNA molecule. This is lower than anticipated given the survey suite of fifteen restriction enzymes commonly employed in this type of study. Across the

specimens examined for mtDNA variation, only five distinct haplotypes were detected. The average haplotypic diversity over all populations was $h = 0.126 \pm 0.004$, accompanied by average nucleotide diversity of $d = 0.004 \pm 0.0001$. Both of these values are low compared to other fishes (Avise 1994). In fact, for the Virginia *E. chaetodon* populations, no mtDNA diversity was detected ($h = 0.000$ and $d = 0.000$); all individuals were characterized by the common mtDNA haplotype (denoted AAA). Therefore, the Virginia populations of blackbanded sunfish did not show a pattern of significant ($P < 0.05$) divergence from one another based on mtDNA haplotype distributions. This absence of genetic variation at the mtDNA locus created some analytical challenges; it was not possible to estimate the levels of diversity and divergence for comparison with other (potentially less impacted) populations and there was little point in constructing a phylogenetic tree. Although the absence of mtDNA variation was unusual, this did not imply that there was no remaining genetic diversity in Virginia populations; merely that this mtDNA survey detected only one maternal lineage.

Measures of genetic variation observed for mtDNA in the North Carolina samples were higher than observed in Virginia ($h = 0.475 \pm 0.028$ and $d = 0.012$). The two subsamples did not differ significantly in mtDNA haplotype frequencies ($P = 0.625$). This southernmost population exhibited nucleotide divergence (d) levels from other populations ranging from 0.002 to 0.013. The New Jersey *E. chaetodon* populations also exhibited higher mtDNA variation ($h = 0.315 \pm 0.219$ and $d = 0.009 \pm 0.007$) than observed for the Virginia populations, and levels of nucleotide divergence (d) ranging from 0.0002 to 0.012. The levels of mtDNA variation detected in North Carolina and

New Jersey populations were comparable to recorded values for other fishes (Avise 1994).

From the four New Jersey populations of blackbanded sunfish, all five haplotypes were recorded, including the common AAA haplotype fixed in Virginia (Table 2). One New Jersey population, Presidential Lake, exhibited only the common genotype, a pattern similar to the Virginia populations. Another New Jersey population, Horicon Lake, exhibited extremely divergent haplotype frequency distributions. Overall, the four New Jersey populations sampled were significantly different from one another ($X^2 = 95.9$; $P < 0.001$) and they exhibited a higher level of mtDNA variation ($h = 0.315 \pm 0.219$ and $d = 0.009 \pm 0.007$) than that observed in the Virginia populations.

Based on mtDNA data, the overall F_{ST} value for all ten blackbanded sunfish populations was 0.4374 ($P < 0.001$). Among the New Jersey populations, average F_{ST} was 0.3790 ($P < 0.001$). The New Jersey populations were found to be significantly different from one another and from the North Carolina population, (X^2 ranging from 13.9 to 42.2; $P < 0.001$ in every case). Using a neighbor-joining clustering algorithm, the differences among populations were clear (Figure 2). Three of the four New Jersey populations clustered tightly with the Virginia populations. The remaining population, Lake Horicon, differed from the New Jersey/Virginia clade by a genetic distance of 0.011. The amount of difference between Lake Horicon and the other nine populations was more than an order of magnitude larger than the next most different pair of populations, Lakes Chatworth and Oswego, which were separated by a genetic distance of $D_s = 0.0008$.

Microsatellite diversity and population differentiation

Six microsatellite loci revealed considerable information regarding genetic diversity within the known Virginia populations and divergence among the Virginia, New Jersey, and North Carolina populations. A total of 85 alleles were detected across the populations, 76 % of which occurred at a frequency of 5% or lower (Table 1). Tests for population differentiation revealed that all populations were significantly different from one another ($P < 0.0001$). Expected and observed heterozygosities (Table 3) were low to moderate in most samples. In Virginia and North Carolina populations, observed heterozygosities were lower than expected in almost all instances, whereas the New Jersey populations exhibited higher observed heterozygosities than expected. In comparison of absolute levels of heterozygosity of Virginia populations to others from New Jersey and North Carolina, the quantity of genetic biodiversity was lower. Testing for heterozygote deficiency further revealed that the Virginia populations were deficient in heterozygotes; whereas New Jersey and North Carolina populations did not demonstrate a lack of heterozygosity. All populations (except 602 and Neblett's Millpond which were not statistically analyzed) showed significant deviation from Hardy-Weinberg equilibrium indicating departures from random mating (Table 3).

Inbreeding coefficients were found to be very high in all of the populations, ranging from 0.23 to 0.76 (Figure 3). These values signify a great deal of inbreeding occurring throughout these populations. *Post hoc* significance testing using ANOVA revealed that populations in the three regions differed significantly based on their inbreeding coefficients ($P < 0.05$).

The average overall value Φ_{ST} for blackbanded sunfish populations was 0.51 ($P <$ 0.0001), indicating that approximately 51 % of the genetic diversity in the total sample was due to differences among the populations - a substantial amount of genetic subdivision. For the Virginia populations, Φ_{ST} was 0.34 compared to 0.53 for New Jersey populations and 0.03 for the North Carolina population (Tables 4 and 5).

The estimated number of effective migrants among population pairs was N_em = 1.3 for Virginia populations and 3.4 among the New Jersey populations (only one North Carolina population). The highest levels of gene flow recorded in this study were estimated among populations in New Jersey (Table 6). Spatial structure elucidated through isolation by distance analysis using the pairwise Φ_{ST} values and corresponding geographic distances, revealed a significant relationship between physical distance and genetic distance ($P < 0.0001$).

Discussion

There is of course, no universal "best" marker or technique for uncovering genetic variation. The mtDNA genetic analysis provided an excellent measure of genetic diversity within and among populations outside Virginia, but detected no genetic variation in the endangered populations of interest. Several explanations may account for the observed lack of mtDNA diversity in Virginia. The simplest explanation is that the marker and approach chosen (mtDNA) failed to uncover fine-scale divergence among the populations. An mtDNA survey using 15 restriction endonucleases would be expected to uncover considerable evidence of fine scale structure if it exists (Epifanio *et al.* 1995).

The patterns observed in the populations outside of Virginia suggest that, if present, a signal would have been detected given the sample sizes assayed. Furthermore, given the intensity of molecular sampling and the lack of mtDNA variation at 12 of the 15 sequences, we conclude that low levels of mtDNA variation are inherent in *E. chaetodon* and that the mtDNA molecule is not sufficiently sensitive to detect fine-scale divergences among Virginia populations. Second, the populations and individuals within them may have had a recent common origin. Although at present they may be geographically isolated, they historically may have experienced considerable female mediated gene flow among local populations; that is, they may represent a common metapopulation. Third, under an expectation of cytonuclear disequilibrium, i.e., in the case that the mtDNA genome is interdependent with the nuclear genome (Asmussen *et al.* 1987), the observation of a single haplotype across the Virginia populations may be a signal that populations were adapted to local conditions and that stabilizing selection had narrowed variation within the populations, as reflected by (but not caused by) mitochondrial genes. A fourth explanation is that populations may be artificially homogenous due to recent mixing, with a single female lineage emerging successful. The probability of this option can be generated using nuclear characters, but given the distribution of the six Virginia blackbanded sunfish populations across at least two watersheds, this scenario is not likely. Lastly, the populations may have experienced such sufficiently strong genetic drift effects that the common variant became fixed; that is, populations were sufficiently small during bottleneck events that rare genotypes were lost.

Data from the New Jersey and North Carolina populations provided a benchmark for determining whether the low mtDNA variability in Virginia populations was an induced phenomenon or characteristic of the species across its range. The high level of mtDNA variation detected for populations outside Virginia shows that Virginia populations are much less variable than some other wild blackbanded sunfish populations. To specifically address the mechanism underlying genetic characteristics of these regions (anthropogenic, vicariant, or a combination of the two forces), three scenarios were considered. First, if North Carolina exhibits low diversity similar to Virginia, the most likely mechanism underlying low genetic variability is natural biogeography. Second, if North Carolina exhibits the same haplotypes and levels of variation similar to New Jersey, then the most likely mechanism is one or more population bottlenecks. Lastly, if North Carolina exhibits more variation than New Jersey or different haplotypes accompanied by similar levels of diversity, then the most likely scenario for genetic structure is a combination of geography and bottlenecks. Given the recent population history of blackbanded sunfish in Virginia, combined with the observation that regions sampled north and south of Virginia exhibited the same mtDNA haplotypes and similar levels of variation, the most likely explanation for the absence of mtDNA variability in the six Virginia populations is one or more severe population bottlenecks.

Nuclear markers alleviated the problem of low mtDNA variation exhibited by blackbanded sunfish populations and satisfied two additional goals. First, the results of a screen from the nuclear genome provided an independent test of patterns observed with

the mtDNA. Second, microsatellites demonstrated great utility in detecting fine-scale population structure, where mtDNA did not. All of the populations deviated from HWE (the exceedingly small 602 and Neblett's Millpond samples were dropped from further statistical analyses). Based on allele frequencies, New Jersey and North Carolina populations exhibited similar levels of variation and supported a more heterogenous mix of alleles which was demonstrated as a greater number of genotypes present in these populations. Additionally, whereas the Virginia populations displayed a pattern bordering on allelic fixation, the New Jersey and North Carolina populations had more typical allele distributions. These results support the contention that Virginia populations are less genetically diverse than either New Jersey or North Carolina and signify that the most likely scenario for the genetic structure observed at microsatellite loci would be a combination of geography and bottlenecks.

Two aspects of the inbreeding estimates are relevant to blackbanded sunfish management. First the degree of inbreeding detected across blackbanded sunfish populations (0.23 - 0.76) is substantial and signifies many generations of sibling mating (brother-sister mating results in $F = 0.125$). Few inbreeding studies in fishes have been conducted and those that do present inbreeding effects (e.g., Tave 1999) indicate levels roughly half the level observed in this study for blackbanded sunfish. Second, the finding that inbreeding levels are significantly higher in Virginia (ranging from 0.55 to 0.72) than they are in populations to the north and south of the region reinforces the notion that Virginia populations are severely impacted and require critical management. Such levels of inbreeding are highly undesirable and signify that measures should be taken to reduce

the amount of inbreeding in these populations.

Convention has it that one migrant per generation is sufficient to maintain gene flow, regardless of population size. The basis of this one-migrant-per-generation rule is the expression that approximates the equilibrium inbreeding coefficient of an ideal population that receives migrants (Slatkin 1987). Both Mills and Allendorf (1996) and Vucetich and Waite (2001) illustrated, however, that for actual non-ideal populations, the required number of migrants is strongly dependent on population size. In the present case, there is potential gene flow between certain of the Virginia populations (with the exception of Harrell's Millpond and Lake Binford). The low levels of migration and gene flow uncovered within the Virginia populations combined with the fact that these populations are highly inbred with small population sizes, suggests that one migrant per generation is not sufficient to sustain these populations.

Management implications

Genetic data such as these are necessary for developing an appropriate management plan and research strategy for *E. chaetodon* in Virginia. The two data types combined provide useful information for deciding which populations should be grouped together as genetic management units. On the one hand, the mtDNA results suggest one or more recent genetic bottleneck (anthropogenic) impacts to *E. chaetodon* in Virginia and a significant amount of geographic substructure. On the other hand, microsatellite results suggest that the bottleneck was not overly severe, and confirm that geographic substructure is a notable characteristic of this species' distribution. Due to the amount of differentiation

detected across populations, $\Phi_{ST} = 0.5151$, treating the species as a single management unit is clearly not biologically warranted. That Virginia populations should be managed as a separate unit is supported by the substantial population divergence estimated for most population comparisons ($F_{ST} \sim 0.4$) and the mtDNA effective female lineage size $N_{eF} = 1$ for Virginia populations. Although the Virginia populations are not significantly different from one another based on their mtDNA distributions, they are different based on their microsatellite allele frequencies. In their present state, the surveyed Virginia populations of blackbanded sunfish, as a unit, are significantly different from the surveyed New Jersey and North Carolina populations ($P < 0.001$), as evidenced by the moderate level of nucleotide sequence divergence, evidence of genetic subdivision, and differences in levels of inbreeding.

Both the Virginia Blackbanded Sunfish Recovery Plan (Smogor *et al.* 1999) and the field survey of blackbanded sunfish distribution in Virginia (Smith *et al.* 2000) recommend consideration of reintroduction into formerly occupied waters within the species' range. Consideration of genetic data is imperative prior to, during, and following any such reintroduction activity. The molecular genetic observations recorded in this study indicate that a reintroduction program, if undertaken, should utilize Virginia stocks and that transfers should be restricted to the level of watershed. Documenting and monitoring the genetic makeup of the donor and recipient populations will allow judicious selection of donor populations (as indicated here from geographically proximal sites) and will provide a means of evaluating reproductive variance and effectiveness of the restoration culture program. Even with the use of genetic data, there is an important

balance to be struck within a restoration-based breeding program. Caution should be taken to avoid deleterious population mixing when transferring individuals or gametes across watersheds or regions. Given the lack of mtDNA variability and the high levels of polymorphism detected using microsatellite DNA, the most reasonable means of determining relatedness among prospective broodstock will be to continue to examine nuclear gene variability.

For the Virginia populations, it will be especially important to minimize the effects of inbreeding by implementing a breeding program that incorporates the use of pedigrees. Furthermore, when captive propagation is undertaken for restoration purposes, microsatellite data will be necessary to assess the adequacy of breeding practices for blackbanded sunfish by providing a means of directly tracing ancestry of individuals, determining reproductive variance among families in the hatchery, and monitoring success of various families following reintroduction to the wild population, a process outlined in detail by Avise (1994). Pedigreed mating for reduced reproductive variance, a valuable tool for supplementing populations, differs from random mating in providing that each male and female can leave more than one descendant, but all fish must leave the same number of offspring. The progeny that become brood fish in the following generation must subsequently be selected randomly from each family (Tave 1999). Through use of pedigreed mating, the number of effective breeders in a population will increase by artificially increasing genetic variance through ensuring that each brood fish is represented in the next generation.

For continued survival of extant blackbanded sunfish populations and eventual reintroduction into historically known areas to succeed, the most important management option at the present time is to maintain and enhance blackbanded sunfish habitat. There are many suitable areas surrounding the current habitat that could theoretically support new populations. Whether habitat is restored through active manipulation or non-action strategies that depend on natural processes, some form of protection is necessary for areas of the Virginia watershed in which blackbanded sunfish still survive if we are to ensure maintenance of biodiversity in this species.

Acknowledgements

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Figure Legends

Figure 1. Known range of *Enneacanthus chaetodon*, blackbanded sunfish, in North America (upper inset), sample collection sites in United States mid-Atlantic region, and detail of collection locations within the Nottoway and Blackwater River Watersheds of the Chowan River Drainage System (lower inset). Eleven sampling locations comprise the eight points shown across New Jersey, Virginia, and North Carolina. Sample sizes in parentheses.

Figure 2. Neighbor-joining tree constructed from Nei's D_S values among ten populations of blackbanded sunfish, *E. chaetodon*. D_S scale shown at bottom left.

Figure 3. Distribution of inbreeding coefficients in *E. chaetodon* populations sampled from three regions of the mid-Atlantic. Frequencies appear on the vertical axis and inbreeding coefficient values along the horizontal axis.

Figure 1.

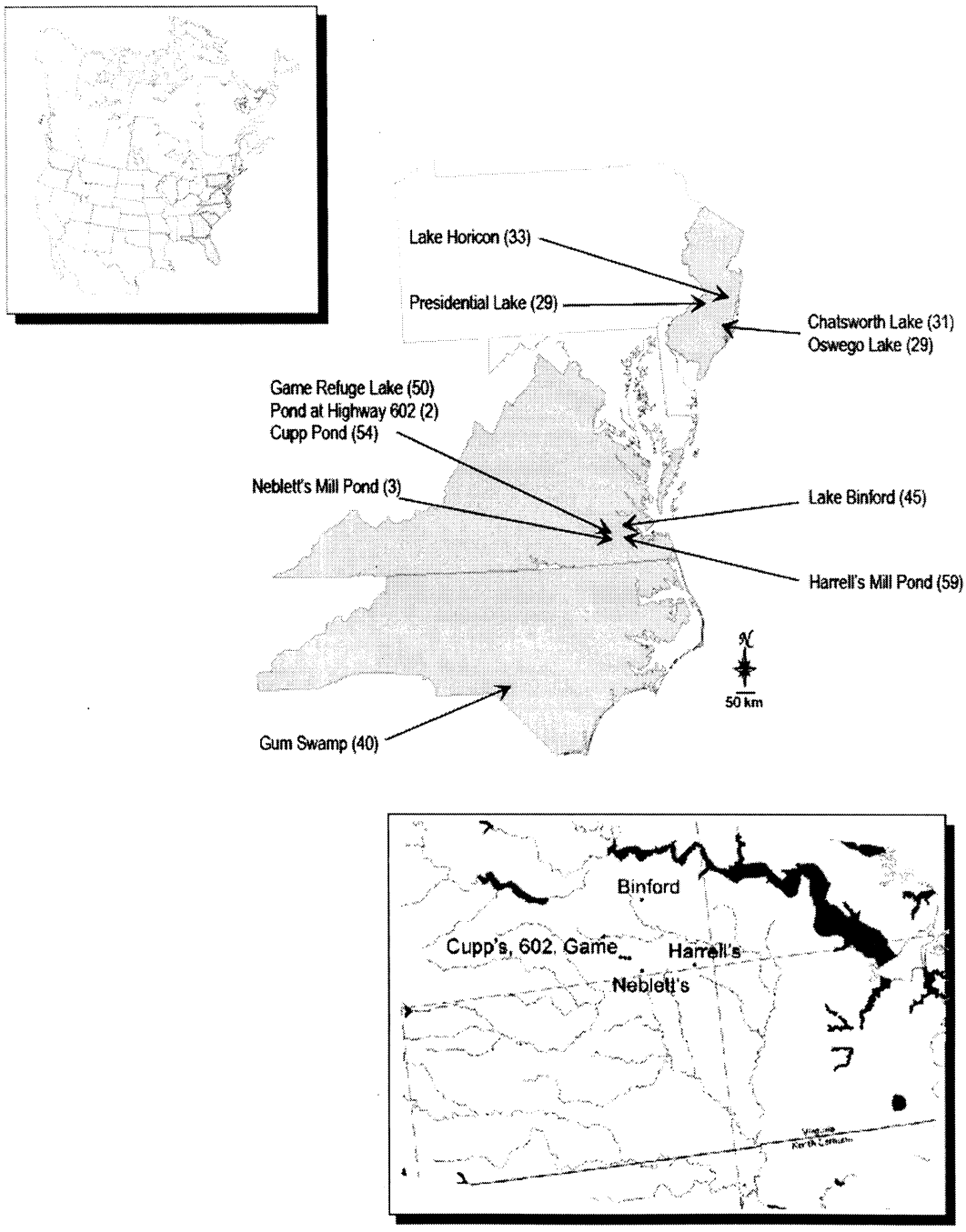
Lake Horicon (33)
Presidential Lake (29)
Chatsworth Lake (31)
Oswego Lake (29)
Game Refuge Lake (50)
Pond at Highway 602 (2)
Cupp Pond (54)
Neblett's Mill Pond (3)
Lake Binford (45)
Harrell's Mill Pond (59)
Gum Swamp (40)
50 km
Binford
Cupp's, 602, Game
Harrell's
Neblett's

Figure 2.

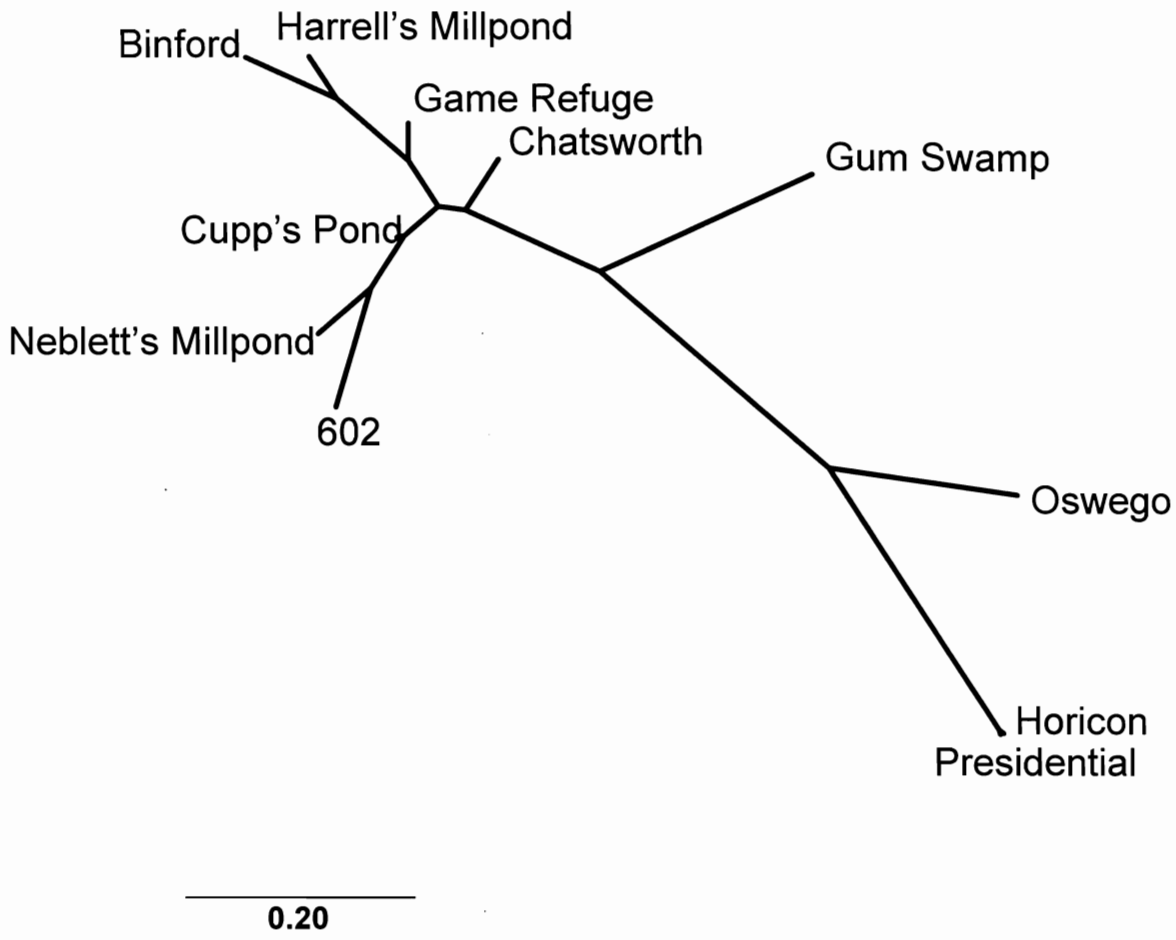
Binford
Harrell's Millpond
Game Refuge
Chatsworth
Gum Swamp
Cupp's Pond
Neblett's Millpond
602
Oswego
Horicon
Presidential
0.20

Figure 3.

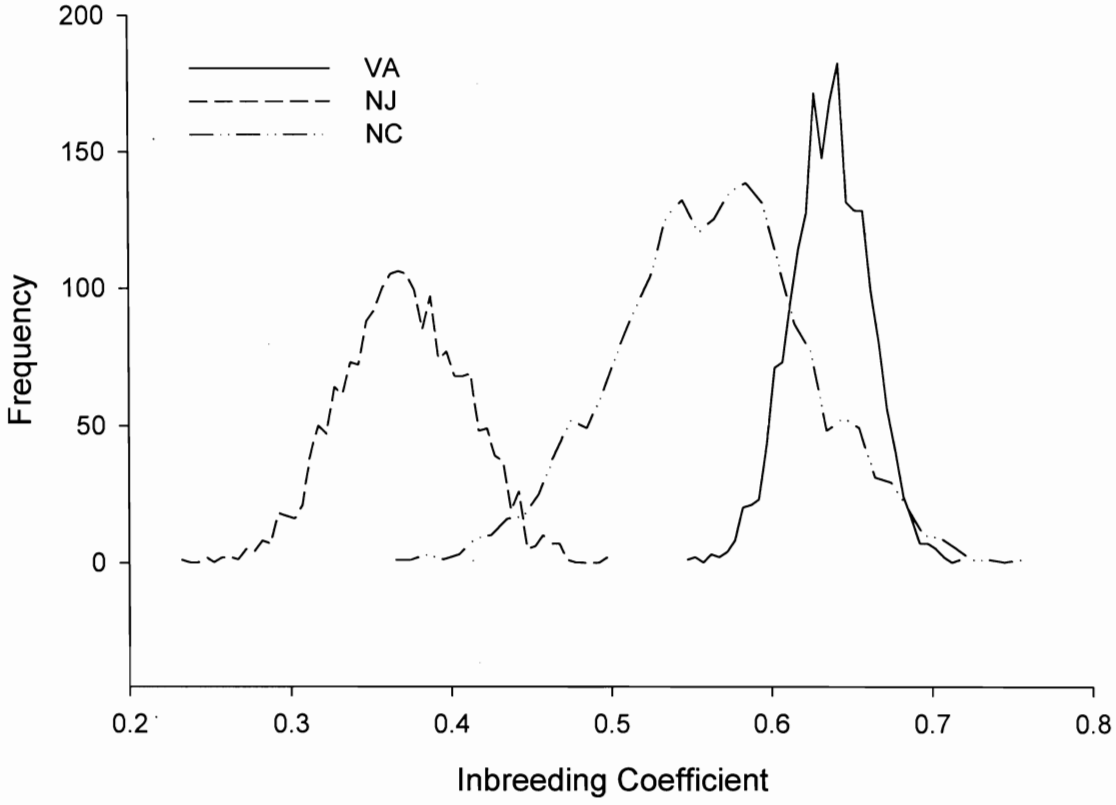
200
150
100
50
0
Frequency
VA
NJ
NC
0.2
0.3
0.4
0.5
0.6
0.7
0.8
Inbreeding Coefficient

Table 1. Primer sequences for three mtDNA segments amplified from *E. chaetodon* and then cleaved with restriction endonucleases, and six microsatellite loci including expected size and observed number of alleles.

MtDNA Segment	Primer sequences forward and reverse	Expected Size Range	Observed no. alleles
cyt b to 16s rRNA	**Ech XL A** 5' ACCCAAATCCTCACAGGCCTCTTTCTTGCT **Ech XL D** 5' CCAACATCGAGGTCGTAAACCCCCTTGTCG	4.5 kb	1
16s rRNA to ATPase 6	**Ech XL C** 5' GGATCAGGACATCCTAATGGTGCAGCCGC **ATPase 2** 5' GACGGCTCATTTATGTCCTCCGGGGCTGAG	6.0 kb	3
ATPase 6 to cyt b	**ATPase 1** 5' CTCGGCCTCCTCCCATATACTTTTACCCCCACTACA **Ech XL B** 5' GGCCAAGCAGAGACCTAGGAGGGAGC	6.5 kb	2
Microsatellite Locus			
Ech9	**F** CAGAGAGTGACAGGCAGACTATAG **R** CCTGTTTCTCTTTCTGTCTCCAAC	92-136 bp	12
Ech12	**F** CCAGCAAAGGTCTGTGTGAC **R** TCACATGCTGCTCACAGTCC	87-119 bp	9
Ech14	**F** GGGCTGCCATTCACATACTTAG **R** TGATGAAAATGCAGAAAGGCCG	114-166 bp	7
Ech32	**F** GAAACATGATGACACTTGATTTATTCT **R** AAATTCATCAGGGCTCCTTAA	120-210 bp	22
Ech33	**F** CCCACTCAATATTATTTCTGTTTACA **R** CTGTGAGCAGGACAAGC	100-160 bp	12
RB20	**F** GGTCTACTGGTAAATGAGGG **R** GTTGGGCTGTCGAGAGTAAAAA	222-294 bp	23

Table 2. Distribution of mtDNA haplotypes among eleven populations of blackbanded sunfish, *E. chaetodon*, sampled from New Jersey, Virginia and North Carolina.

	New Jersey				Virginia						No. Carolina
	Chatsworth Lake	Oswego Lake	Presidential Lake	Horicon Lake	Pond Above County Road 602	Lake Binford	Cupp Pond	Game Refuge Lake	Harrell's Millpond	Neblett's Millpond	Gum Swamp
AAA	22	23	29	11	2	29	52	36	41	2	25
AAH	2	0	0	0	0	0	0	0	0	0	15
AEA	0	0	0	22	0	0	0	0	0	0	0
DAA	4	6	0	0	0	0	0	0	0	0	0
DAH	3	0	0	0	0	0	0	0	0	0	0
	31	29	29	33	2	29	52	36	41	2	40

Table 3. MtDNA and microsatellite diversity and divergence indices for each population of *E. chaetodon* sampled from New Jersey, Virginia, and North Carolina. Sample sizes are shown in parentheses next to each population. Number of haplotypes, haplotype diversity (*h*) and nucleotide diversity (Π) are shown for mtDNA data. The average observed and expected heterozygositites (H_O and H_E) and the Hardy-Weinberg Equilibrium Chi-square associated *P*-values are shown for the micorsatellite data. Populations are those shown in Figure 1 and are abbreviated as follows: CL, Chatsworth Lake; CP, Cupp Pond; GR, Game Refuge Lake; GS, Gum Swamp Lake; HL, Horicon Lake; HM, Harrell's Millpond; LB, Lake Binford; OL, Oswego Lake; and PL, Presidential Lake.

	MtDNA diversity and divergence			Microsatellite diversity		
Pop. (n)	# Haplo	*h* (± sd)	Π	H_O	H_E	*P*-value
CL (26)	4	0.4738 ± 0.0703	0.0157	0.3145	0.3375	< 0.0001
CP (54)	1	0.0000 ± 0.0000	0.0000	0.3987	0.5701	< 0.0001
GR (50)	1	0.0000 ± 0.0000	0.0000	0.2613	0.3537	< 0.0001
GS (40)	2	0. 4750 ± 0.028	0.0120	0.3997	0.4468	< 0.0001
HL (28)	2	0.4513 ± 0.0399	0.0118	0.2357	0.2095	< 0.0001
HM (59)	1	0.0000 ± 0.0000	0.0000	0.3660	0.5855	< 0.0001
LB (45)	1	0.0000 ± 0.0000	0.0000	0.3098	0.5380	< 0.0001
OL (25)	2	0.3339 ± 0.0629	0.0087	0.5387	0.4408	< 0.0001
PL (25)	1	0.0000 ± 0.0000	0.0000	0.2272	0.1623	< 0.0001

Table 4. Fixation indices and probability of significance (in parentheses) among *E. chaetodon* populations surveyed in New Jersey, Virginia, and North Carolina. Above the diagonal are estimates of F_{ST} based on mtDNA data. Below the diagonal are estimates based on microsatellites for Φ_{ST}. Note: F_{ST} is undefined for the Virginia populations because no genetic variability was detected using mtDNA.

	Population								
	Chatsworth	**Oswego**	**Presidential**	**Horicon**	**Cupp**	**Game**	**Binford**	**Harrell's**	**Gum**
Chatsworth		0.017 (0.346)	0.178 (0.000)	0.471 (0.000)	undef	undef	undef	undef	0.120 (0.000)
Oswego	0.342		0.179 (0.000)	0.531 (0.000)	undef	undef	undef	undef	0.276 (0.000)
Presidential	0.793	0.261		0.641 (0.000)	undef	undef	undef	undef	0.321 (0.000)
Horicon	0.783	0.219	0.046		undef	undef	undef	undef	0.534 (0.000)
Cupp	0.189	0.344	0.667	0.654		undef	undef	undef	undef
Game Refuge	0.409	0.490	0.822	0.811	0.265		undef	undef	undef
Binford	0.464	0.503	0.762	0.745	0.410	0.411		undef	undef
Harrell's	0.403	0.427	0.696	0.677	0.252	0.416	0.268		undef
Gum Swamp	0.430	0.384	0.702	0.690	0.393	0.538	0.551	0.489	

Table 5. ANOVA and AMOVA results for New Jersey, Virginia, and North Carolina populations of *E. chaetodon* including the F_{ST} and Φ_{ST} values, respectively, associated with populations (and population subsamples). Results include degrees of freedom (df), sum of squares (SS), and significance values (*P*-value).

ANOVA	Source	SS	df	F_{ST}	*P* - value
	Among regions	75.289	2	20228.441	0.000
	Within regions	11.162	5998		
	Total	86.451	6000		
AMOVA	**Source**	**SS**	**df**	Φ_{ST}	***P* - value**
NJ	Among populations	105.857	3	0.405	0.000
	Within populations	188.825	100		
	Total	294.683	103		
VA	Among populations	120.402	5	0.252	0.000
	Within populations	419.523	207		
	Total	539.925	212		
NC	Among samples	1.520	1	-0.013	0.684
	Within samples	74.429	37		
	Total	75.949	38		

Table 6. Pairwise effective migration rate, N_em, computed by the standard relationship of N_em to F_{ST}.

	Population								
	Cupp	**Game**	**Binford**	**Harrel's**	**Chatsworth**	**Oswego**	**Presidential**	**Horicon**	**Gum**
Cupp									
Game	1.9								
Binford	1.5	inf							
Harrel's	2.8	5.3	2						
Chatsworth	0.9	1.4	1.2	1.2					
Oswego	1.2	3.2	3.1	1.9	1.3				
Presidential	1.1	1.7	2.3	1.5	1.3	2.2			
Horicon	0.5	0.7	0.7	0.6	0.6	0.7	3.2		
Gum	1.2	6.7	inf	1.9	1.1	2	1.8	0.6	

Vita

Diana Marie Kercher was born February 6, 1979, in Newport News, Virginia. She attended Newport News Public Schools and graduated from Menchville High School in 1997. Upon graduation she moved to Richmond, Virginia and matriculated at Virginia Commonwealth University, where she earned her Bachelors of Science in Biology. After obtaining her undergraduate degree, she was accepted into the Masters program at VCU and conducted her research in Dr. Bonnie Brown's lab.