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Genetic assessment of blackbanded sunfish in Virginia

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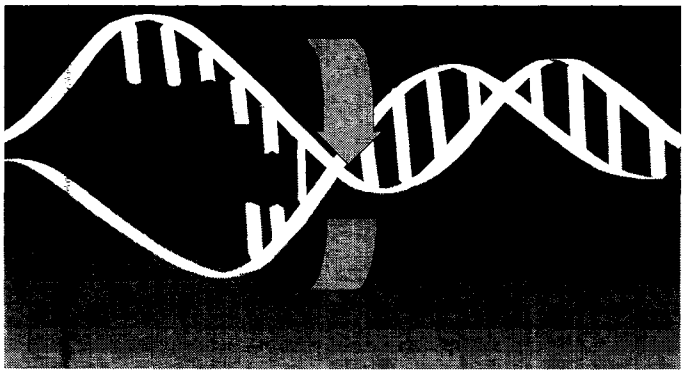

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Executive Summary

Previously, a survey of mitochondrial DNA (mtDNA) genetic variation was completed for blackbanded sunfish, *Eneacanthus chaetodon*, from six known populations in Virginia and four comparative populations in New Jersey. The total sample exhibited lower levels of mtDNA variation than generally observed for other centrarchids and the Virginia populations were devoid of detectable mtDNA variation. The pattern of genetic variation uncovered by mtDNA indicated that Virginia populations had likely undergone one or more severe population bottlenecks. We subsequently examined nuclear DNA microsatellite markers for the existing population samples as well as an additional sample from North Carolina (examined for both mtDNA and microsatellite variability). The total sample was characterized by a high degree of genetic variation. All populations, including those sampled in Virginia, exhibited moderate to high levels of heterozygosity, differentiation, and gene flow. Microsatellite data indicated that Virginia populations are about as genetically variable as other blackbanded sunfish populations across the sampled range, indicating that the past bottlenecks did not render Virginia populations genetically depauperate. Genetic variation at both characters exhibited significant geographic structuring across the range sampled (New Jersey, Virginia, and North Carolina). For Virginia, the combination of a lack of detectable mtDNA variation, high levels of microsatellite variation, geographic differences between Virginia populations and other regions, and high levels of gene flow, indicated that blackbanded sunfish populations from this region are different from sunfish in other regions. The primary implications of these data for sunfish restoration plans are that careful attention must be paid to avoiding inbreeding in any artificially reared populations destined for release and that only Virginia stocks, preferably those from the same watershed, should be used for obtaining breeders for a supplementation program.

Introduction

This report outlines the cumulative results of a genetic survey of blackbanded sunfish, *Enneacanthus chaetodon*, in Virginia and in comparable regions to the north and south. The data are provided to support decisions regarding management options to facilitate blackbanded sunfish recovery. The project was a joint effort of VDGIF and VCU scientists to conduct field and genetic surveys in compliance with Virginia's Blackbanded Sunfish Recovery Plan.

Information on the degree to which Virginia populations differ from one another and from more distant populations was collected to anchor our expectations for sunfish recovery. The first question addressed in Phase I was, "Are the Virginia populations of blackbanded sunfish different enough from each other to require keeping them separate?" This question required that we simultaneously address another issue, "Are the Virginia populations distinct from other populations of *E. chaetodon* to the north (e.g., New Jersey) or the south (e.g., North Carolina)?" The third question focused on whether captive-bred population(s) exhibited sufficient genetic diversity to prove effective for supplementing the declining numbers of wild blackbanded sunfish. A final reference for genetic comparison included two closely related species, the blue spotted sunfish, *E. gloriosus*, and the banded sunfish, *E. obesus*, which also occur in Virginia. If the relict *E. chaetodon* populations in Virginia were found not to be significantly divergent from one another and if the cultivated population(s) had a sufficiently high level of genetic diversity, release of cultivated fish would be an effective method of supplementing their declining numbers. However, a finding of significant divergence among the remaining Virginia populations (relative to divergence observed among the Virginia populations and those from other states), would have indicated that the most prudent recovery strategy would be to maintain separate supplementation programs for each Virginia population.

Null hypotheses evaluated

1. Virginia populations of *E. chaetodon* are not significantly divergent from distant populations (specifically, those in New Jersey and North Carolina).
2. Virginia populations of *E. chaetodon* are not significantly divergent from one another (relative to divergence observed among other similarly distributed populations in New Jersey).

Methods

Blackbanded sunfish specimens

Specimens were collected at eleven sites: six in Virginia, four in New Jersey, and one site in North Carolina (Figure 1). The Virginia sites were: Harrell's Millpond; Lake Binford; Cupp Pond, a private pond on the south branch of Dobie Swamp above Game Refuge Lake; "602," referring to the pond off Sussex County Road 602 on Dobie Swamp above Game Refuge Lake; Game Refuge Lake; and Neblett's Millpond on Dobie Swamp below Game Refuge Lake. The specific coordinates of each Virginia site are listed by Smith et al. (2000). New Jersey collection sites were Chatsworth, Horicon, Oswego and Presidential Lakes. The site surveyed in North Carolina was Gum Swamp Creek. Depending on the field conditions at each site (depth, DO, vegetation, snags, etc.), fish were captured using appropriate gear which included minnow traps, dip nets, seines, and electrofishing. In most cases, tissue samples consisted of non-lethal fin clips. Each fin clip, approximately 1 cm in length, was archived in a separately labeled tube of 70-95% ethanol and held at ambient temperature until the time of DNA extraction.

DNA preparation

DNA was isolated from fin clip samples using a modified STE extraction procedure (Hillis et al. 1996). Specifically, the fin clip was homogenized by hand using a plastic pestle in a 1.5 ml microfuge tube containing 0.5 ml of STE homogenization buffer (0.1 M NaCl, 0.05 M Tris-HCl, pH 7.5, 0.001 M disodium EDTA), 75 µl of 20% SDS, and 12.5 µl of 20 mg/ml proteinase K. Following incubation at 60°C for 2 hr, an equal volume of PCI (25:24:1 phenol:chloroform:isoamyl alcohol) was added, and the mixture vigorously shaken by hand. Following centrifugation for 5 min at 10,000 x g, the upper aqueous layer was removed to a new tube and treated for 30 minutes by addition of 1 µl of a 10 mg/ml RNAse A solution. Each sample was again extracted with PCI and similarly extracted with CI (24:1 chloroform:isoamyl alcohol). The aqueous phase was removed to a new tube and combined with 1/10 volume of 3 M sodium acetate plus 2.5 volumes cold 95% ethanol. Following gentle mixing to foster precipitation of the DNA, the tube was held at –20°C overnight to complete DNA precipitation. DNA was collected by centrifuging for 20 min at 10,000 x g, washed twice with 70% ethanol, and dried in a 37°C oven for one hour. Following resuspension of the DNA pellet in 100 µl of

0.1X TE, samples were held at –20°C. Extracted DNA consisted largely of fragments >10,000 bp and was quantified by spectrophotometry.

Mitochondrial DNA genotyping

Each newly collected specimen from North Carolina was examined for its mitochondrial DNA (mtDNA) haplotype to complete the Phase I genetic biodiversity database. To accomplish mitochondrial DNA genotyping, three large regions collectively covering the entire mitochondrial genome were amplified by methods described in the Phase I Report. Restriction enzyme assays were performed as described in Phase I for the three informative enzymes (*Dra*I, *Eco*RI, and *Hin*dIII). 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 the statistical analysis. Data were analyzed with the REAP statistical package to generate estimates within and among populations of evolutionary distance (Nei, 1987, eq. 5.55), haplotype (Nei 1987; eqs. 8.4, 8.5 and 8.12) nucleotide diversity (Nei, 1987, eqs. 10.19, 10.7, 10.20, 10.21), and divergence (Nei and Tajima 1981). Bootstrapping and phylogenetic tree analysis was done using PHYLIP.

Microsatellite genotyping

Two populations (New Jersey samples from Presidential and Horicon Lakes) were omitted from the microsatellite analyses due to lack of sufficient funds to conduct the necessary analyses. Microsatellites were amplified from each individual in the remaining nine populations using touchdown conditions (i.e., the first 10 cycles had the annealing temperature reduced by 1°C / cycle). All reactions contained 1 µl of 1:10 diluted DNA, 0.16 mM spermidine, 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 (Table 1; one primer in each pair was fluorescently-labeled with either FAM, HEX, or TET), and 1 unit Amplitaq Gold ™ (Applied Biosystems). The completed reactions were purified and resolved on an ABI Prism ™ capillary electrophoresis DNA analyzer. Scoring was facilitated with Genotyper 2.1 software. The genetic data were analyzed with GENEPOP (Raymond and Rousset 1995) to obtain F_{ST}, linkage, and Hardy-Weinberg estimates. Nei's standard genetic distance (D_S; Nei 1987) was calculated for each population pair using

MICROSAT (Minch 1997). PHYLIP software (Felsenstein 1993) was used to obtain a consensus neighbor-joining tree based on 100 bootstrapped D_S value data sets.

Phase II Results

Specimens

During Summer and Fall 1999, samples were collected by Ryan Smith (VDGIF) from each of six *E. chaetodon* populations located in Virginia: Harrell's Millpond (n = 59), Lake Binford (n = 15), Cupp Pond (n = 23; a private pond on the south branch of Dobie Swamp above Game Refuge Lake), "602" (n = 2; referring to the pond off Sussex County Road 602 on Dobie Swamp above Game Refuge Lake), Game Refuge Lake (n = 88), and Neblett's Millpond (n = 3; on Dobie Swamp below Game Refuge Lake). The former two sites are not physically connected to one another or to the Game Refuge series. The latter four potentially experience one-way gene flow from Game Refuge Lake. Additional "outgroup" samples were collected by Dave Littlehale (New Jersey Aquarium) in early October 1999 from four native *E. chaetodon* populations in New Jersey: Chatsworth Lake (n= 35), Oswego Lake (n = 35), Presidential Lake (n = 36), and Horicon Lake (n = 35) and from one population each of *E. gloriosus* (n = 3) and *E. obesus* (n = 5) located in New Jersey. Reference samples were collected from North Carolina by Charlene Couch (NC State) and colleagues at Gum Swamp Creek (n=39) in March and November 2001.

Genetic Analyses

MtDNA survey

Across all specimens, only five distinct mtDNA haplotypes were detected. The average haplotypic diversity over all eleven populations was $\bar{h} = 0.158 \pm 0.004$, accompanied by average nucleotide diversity among the eleven populations of $d = 0.004 \pm 0.000$. Both of these values are low compared to other fishes (Avise 1994 and references therein). As previously reported, the Virginia *E. chaetodon* populations exhibited no detectable mtDNA diversity ($\bar{h} = 0.000$ and $d = 0.000$) and therefore, the Virginia populations of blackbanded sunfish did not show a pattern of significant divergence from one another based on mtDNA haplotype distributions. The absence of genetic variation at the mtDNA locus created some analytical challenges. For example, it was not possible to estimate levels of diversity and divergence for comparison with other (potentially less impacted) populations. Nor was it possible to estimate the fixation index, F_{ST}, among Virginia population pairs because F_{ST} is calculated by examining the patterns of interpopulation

variance. Although the absence of mtDNA variation is a rather unusual finding, we caution that this should not be interpreted to indicate that there is no remaining genetic diversity in Virginia populations; merely that the mtDNA portion of our survey did not detect more than one maternal lineage (i.e., the Virginia populations are estimated to have an effective female lineage size, N_{eF}, of one).

Measures of genetic variation observed for mtDNA in the North Carolina sample were higher than observed in Virginia ($\bar{h}$ =0. 475 $\pm$ 0.028 and d = 0.012). 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 ($\bar{h}$=0.315 $\pm$ 0.219 and d = 009 $\pm$ 0.007) than observed for the Virginia populations, and levels of nucleotide divergence (D) ranging from 0.0002 to 0.012. 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). The levels of mtDNA variation detected in North Carolina and New Jersey populations were comparable to recorded values for other fishes (Avise 1994). As such, these data provided a benchmark for determining whether the low mtDNA variability in Virginia populations is primarily anthropogenic or characteristic of the species across its range. These results showed that Virginia populations were much less variable than other wild blackbanded sunfish populations.

Several explanations may account for the observed lack of mtDNA diversity in Virginia. First, the populations and individuals within them may have a recent common origin. Although at present they may be geographically isolated, they historically experienced considerable female mediated gene flow among local populations; that is, they represent a common "metapopulation." Second, there is an expectation that the mtDNA genome is interdependent with the nuclear genome (called cytonuclear disequilibrium, *sensu* Asmussen et al. 1987). Therefore, the observed single haplotype across the Virginia populations might be a signal that populations are adapted to local conditions and that stabilizing selection has narrowed variation within the populations, and is reflected in (but not caused by) the mtDNA molecule. A third explanation is that populations may be artificially homogenized due to recent mixing with a single female lineage becoming successful. The probability of this option can be examined using nuclear (in this case, microsatellite) characters, but given the distribution of the six Virginia blackbanded sunfish populations across at least two watersheds, this scenario is not likely.

Fourth, the mtDNA assay failed to uncover fine-scale divergences among the populations. Fifth, the populations at issue may have experienced sufficiently strong bottlenecks followed by genetic drift effects that the common variant became fixed; that is, populations were sufficiently small during bottleneck events that rare genotypes were lost. To specifically address the mechanism(s) underlying the genetic patterns observed for mtDNA, we considered the regional data: New Jersey, Virginia, and North Carolina. If variability decreased with longitude, then a likely explanation of the low diversity in Virginia would be vicariance (a circumstance of the last glacial period). If the northern and southern regions exhibited the same haplotypes and similar levels of variation, then the most likely mechanism for reduced variation in Virginia would be one or more population bottleneck(s). If the North Carolina sample exhibited more variation than New Jersey or different haplotypes accompanied by similar levels of diversity, then the most likely scenario for the genetic structure observed would be a combination of geography and bottlenecks. Thus, given the recent population history of blackbanded sunfish in Virginia cited in the Recovery Plan, 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 before the populations dispersed into their present localities and became isolated.

***Table 1. Primer sequences for six microsatellites assayed for* E. chaetodon.**

Name	Primer sequences forward (-F) and reverse (-R)
Ech9-F	5' CAG AGA GTG ACA GGC AGA CTA TA
Ech9-R	5' CCT GTT TCT CTT TCT GTC TCC AAC
Ech12-F	5' CCA GCA AAG GTC TGT GTG AC
Ech12-R	5' TCA CAT GCT GCT CAC AGT CC
Ech14-F	5' GGG CTG CCA TTC ACA TAC TTA G
Ech14-R	5' TGA TGA AAA TGC AGA AAG GCC G
Ech32-F	5' GAA ACA TGA TGA CAC TTG ATT TAT TCT
Ech32-R	5' AAA TTC ATC AGG GCT CCT TAA
Ech33-F	5' TCC CAC TCA ATA TTA TTT CTG TTT ACA
Ech33-R	5' CTG TGA GCA GGA CAA GC
RB20-F	5' GGT CTA CTG GTA AAT GAG GG
RB20-R	5' GTT GGG CTG TCG AGA GTA AAA A

Figure 1. Map showing approximate locations of* E. chaetodon *collection sites in New Jersey, Virginia, and North Carolina.

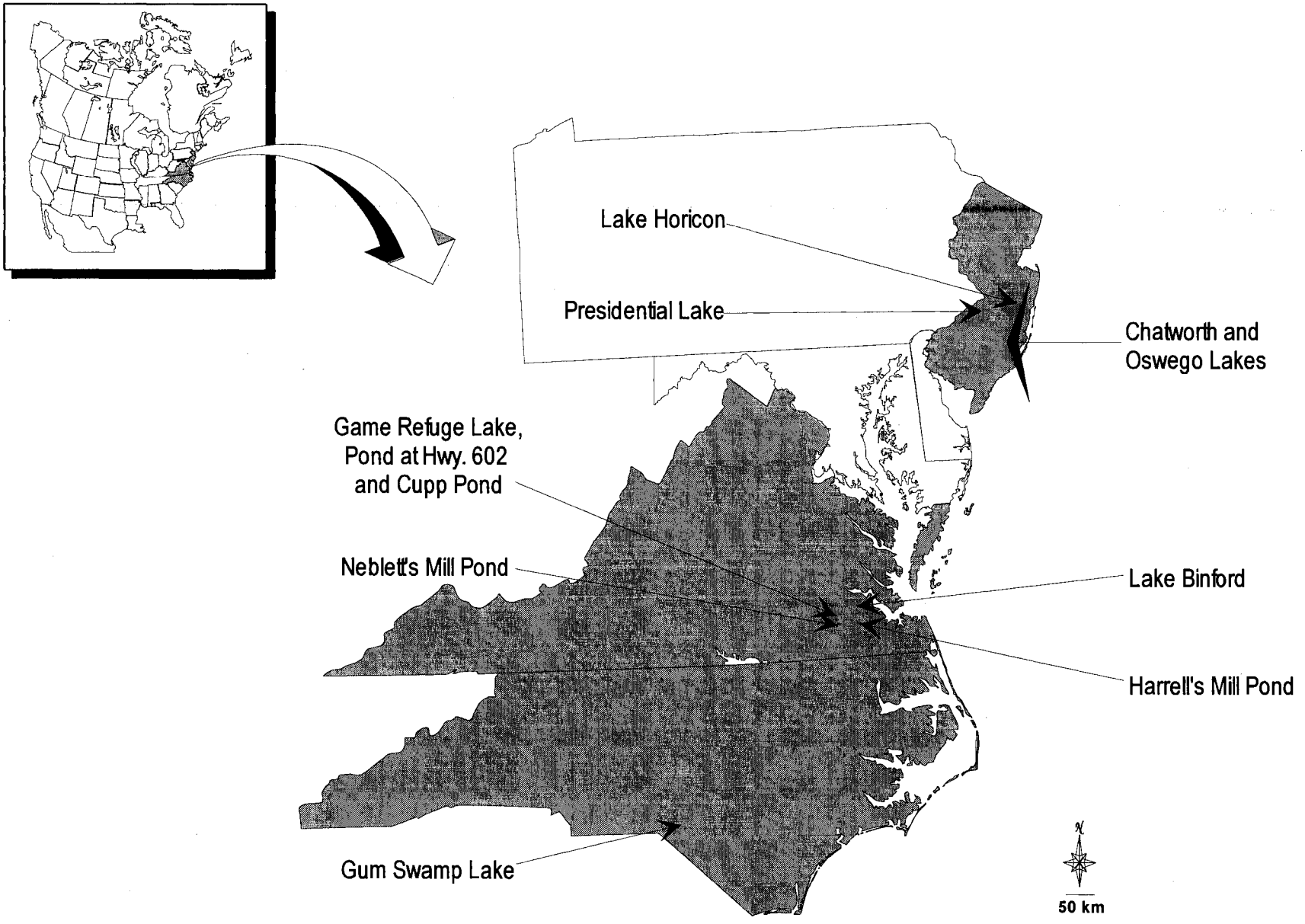


Table 2. Distribution of mitochondrial DNA haplotypes among eleven populations of blackbanded sunfish sampled in New Jersey, Virginia, and North Carolina.

	New Jersey				Virginia						North 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 Creek
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. Fixation index of inbreeding, F_{ST}, estimated using mtDNA data for blackbanded sunfish populations surveyed in New Jersey and North Carolina followed by the probability of significance in parentheses. F_{ST} is undefined for the Virginia populations because no genetic variability was detected using mtDNA.

	Oswego	Presidential	Horicon	Gum
Chatsworth	0.017 (0.254)	0.178 (0.000)	0.471 (0.000)	0.120 (0.000)
Oswego		0.179 (0.000)	0.531 (0.000)	0.276 (0.000)
Presidential			0.641 (0.000)	0.321 (0.000)
Horicon				0.534 (0.000)

Figure 2. Neighbor-joining tree constructed from Nei's evolutionary distance (D) values derived from mtDNA haplotypes frequencies in eleven populations of* E. chaetodon *(the abbreviation "Pop11" refers to the Gum Swamp population from North Carolina).

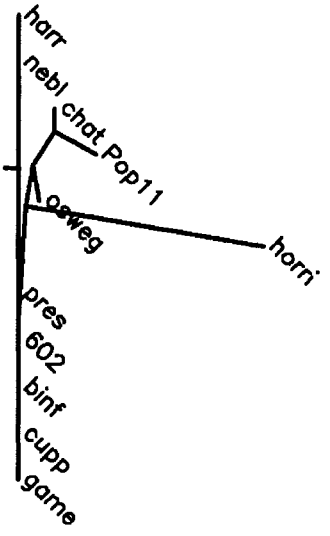


Table 4A. Sample sizes (n), numbers of alleles (A) for each microsatellite, and observed (H_o) and expected (H_e) heterozygosities in Eneacanthus chaetodon populations from New Jersey (Chatworth and Oswego), Virginia (602, Binford, Cupp's, Game, Harrell's and Neblett's), and North Carolina (Gum Swamp Creek).

	Locations										
	NJ		VA						NC		
Locus	Chatworth	Oswego	602	Binford	Cupp	Game	Harrell's	Neblett's	Gum	Total	Mean
n	26	10	2	45	54	50	59	3	39	288	32
Ech9											
A	2	1	2	6	7	3	5	2	9	12	4.1
H_o	0.00	--	0.00	0.00	0.04	0.06	0.08	0.00	0.00		0.02
H_e	0.31	--	0.50	0.53	0.41	0.10	0.20	0.67	0.59		0.41
Ech12											
A	2	2	1	3	5	2	6	1	2	9	2.7
H_o	0.04	0.00	--	0.02	0.04	0.02	0.07	--	0.02		0.03
H_e	0.04	0.20	--	0.04	0.11	0.02	0.41	--	0.02		0.12
Ech14											
A	1	1	2	2	3	2	3	2	2	6	2.0
H_o	--	--	0.50	0.36	0.33	0.32	0.10	0.00	0.23		0.26
H_e	--	--	0.50	0.47	0.41	0.38	0.15	0.67	0.28		0.41
Ech32											
A	8	7	1	7	5	4	7	2	3	19	4.9
H_o	0.96	0.70	--	0.22	0.04	0.26	0.14	0.00	0.20		0.32
H_e	0.85	0.80	--	0.29	0.35	0.58	0.44	0.67	0.28		0.53
Ech33											
A	2	4	1	6	6	2	4	2	3	11	3.3
H_o	0.00	0.10	--	0.22	0.07	0.04	0.14	0.00	0.00		0.07
H_e	0.08	0.40	--	0.31	0.33	0.04	0.27	0.67	0.20		0.29
RB20											
A	4	3	4	7	7	8	10	3	9	23	6.1
H_o	0.50	0.20	1.00	0.09	0.33	0.54	0.07	0.33	0.46		0.39
H_e	0.46	0.60	1.00	0.18	0.55	0.66	0.39	0.67	0.67		0.58

Table 4B. Averages for basic microsatellite genetic variation parameters.

	Population Averages									
	NJ		VA						NC	
	Chatworth	Oswego	602	Binford	Cupp	Game	Harrell's	Neblett's	Gum	Mean
A	3.2	3.0	1.8	5.2	5.5	3.5	5.8	2.0	4.7	3.9
H_o	0.30	0.25	0.50	0.15	0.14	0.21	0.10	0.07	0.15	0.19
H_e	0.35	0.50	0.67	0.30	0.36	0.30	0.31	0.67	0.34	0.40

Table 4C. Basic microsatellite genetic variation parameters averaged by region.

	Regional Averages							
	All Pops	sd	New Jersey	sd	Virginia	sd	North Carolina	sd
A	3.9	2.5	3.5	2.3	4.0	4.1	4.7	3.4
H_o	0.19	0.24	0.28	0.36	0.17	0.16	0.15	0.18
H_e	0.40	0.24	0.42	0.29	0.41	0.38	0.34	0.25

Microsatellite survey

More than 30 microsatellites were isolated from blackbanded sunfish and other centrarchids. Each microsatellite locus was evaluated to determine levels of polymorphism and the six most variable were used to survey n = 356 blackbanded sunfish. For these, complete data were obtained for 288 specimens. These six loci uncovered considerable information regarding genetic diversity within the six known Virginia populations and divergence among the Virginia and comparative outgroup populations. The six loci were highly polymorphic in populations of *E. chaetodon* from all three regions (Table 4A). Two of the possible locus pairs exhibited significant linkage disequilibrium (Ech32 *vs.* Ech33 and Ech9 *vs.* RB20; each $X^2 = \infty$; $P < 0.0001$). The mean total number of alleles per population ranged from A = 2 in Population-602 to A = 6 in Cupp Pond. Average expected heterozygosities were moderate in most samples, ranging from 0.30 to 0.67 (Table 4B). Most populations showed significant deviation from Hardy-Weinberg equilibrium at one or more locus ($P < 0.0001$). With a total of 66 tests, only 3 populations were expected to deviate from Hardy-Weinberg equilibrium by chance at $P = 0.05$, yet the deviations for each population still were significant after Bonferroni adjustment. Comparing the levels of heterozygosity for Virginia populations to the total sample and to the subset of populations sampled north (New Jersey) and south (North Carolina) (Table 4C), the quantity of genetic biodiversity was not detectably different.

Because of the exceedingly small sample sizes for Population 602 and Neblett's Millpond, those two populations were dropped from further analyses. Allele frequencies among the seven remaining population samples were not homogenous ($X^2 = \infty$; $P < 0.0001$), indicating significant genic differentiation among sites. In fact, every population pair exhibited high levels of differentiation (Table 5), even with Bonferroni correction. Genetic subdivision using microsatellite data to estimate *F*-statistics yielded an average overall value for the seven blackbanded sunfish populations of $F_{ST} = 0.408$ ($P < 0.001$); indicating that approximately 40% of the genetic diversity in the total sample was due to differences among the populations - a substantial amount of genetic subdivision. F_{ST} values among the Virginia populations ranged from 0.18 to 0.34 indicating moderate genetic subdivision within Virginia. By comparison, F_{ST} was 0.04 between the two New Jersey populations, reflecting the fact that they are in the same watershed and potentially experience high levels of gene flow. Between North Carolina and New Jersey, F_{ST} ranged from 0.12 to 0.53; levels of subdivision similar to those recorded among Virginia populations.

Using the average overall F_{ST} to estimate migration yielded N_em of 0.36 migrants per generation. However, some of the highest levels of gene flow recorded in this study (N_em values as high as 3.6) were estimated among Virginia populations. By convention, $N_em > 1$ is considered sufficient to maintain genetic homogeneity over the segregating influence of mutation and drift, whereas lower levels of N_em are expected to promote population heterogeneity (Slatkin 1987).

Table 5. Chi-square values calculated among seven* E. chaetodon *populations (above diagonal) and the associated* P*-values (below diagonal).

	New Jersey		Virginia				N. Carolina
	Chatworth	Oswego	Binford	Cupp	Game	Harrell's	Gum
Chatworth		40.64	∞	∞	∞	∞	∞
Oswego	<0.0001		∞	∞	∞	∞	∞
Binford	<0.0001	<0.0001		65.52	∞	∞	∞
Cupp	<0.0001	<0.0001	<0.0001		∞	∞	∞
Game	<0.0001	<0.0001	<0.0001	<0.0001		∞	∞
Harrell's	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		∞
Gum	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	

***Table 6. Pairwise estimates of* F_{ST} *based on microsatellite data for populations of* E. chaetodon.**

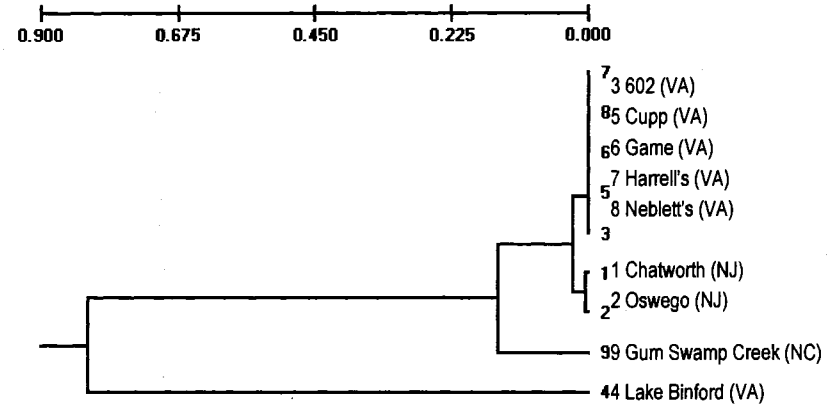
	New Jersey	Virginia				N. Carolina
Population	Oswego	Binford	Cupp	Game	Harrell's	Gum
Chatworth	0.0413	0.4085	0.1572	0.2908	0.4052	0.3721
Oswego		0.3868	0.1188	0.2478	0.3692	0.3493
Binford			0.3425	0.2626	0.2236	0.4641
Cupp				0.1802	0.2319	0.3423
Game					0.3011	0.4153
Harrell's						0.4404

Table 7. Effective migration rates (*N_em*) among seven populations of* E. chaetodon *from New Jersey to North Carolina.

	Chatworth	Oswego	Binford	Cupp's	Game	Harrell's	Gum
Chatworth		0.7326	0.6620	0.9332	0.5017	0.4910	0.2729
Oswego			0.5392	1.2057	0.6970	0.4966	0.3086
Binford				0.7486	0.5869	2.6225	0.2851
Cupp's					3.6819	1.0326	0.3280
Game						0.9872	0.2587
Harrell's							0.2677

Using a neighbor-joining clustering algorithm, a clear geographic relationship emerged based on genetic distances (D_S) among populations (Figure 3). With the exception of Lake Binford, Virginia populations clustered together, as did New Jersey populations, and the North Carolina population was separate from the other two groups. This finding, along with the significant differences observed among populations, argues strongly for separate management of these groups and avoidance of mixing of what are by all accounts clearly divergent units.

***Figure 3. Neighbor-joining tree constructed from Nei's genetic distance (D_S) estimated from microsatellite allele frequencies in nine populations of* E. chaetodon.**



Genetic biodiversity estimates and reintroduction recommendations

Geographic heterogeneity estimates were highly significant, meaning that the blackbanded sunfish populations examined are not part of a single "metapopulation." In addition, F_{ST} values (which signify the level of population divergence due to local inbreeding), were substantial across all samples as well as within both the New Jersey and the Virginia groups of populations. Therefore, treating the species as a single management unit is clearly not genetically warranted.

Combination of the two data types provides 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 substructuring. On the other hand, microsatellite results suggest that the bottleneck was not overly severe, and confirm that geographic substructuring is a notable characteristic of this species' distribution.

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 and evidence of genetic subdivision. 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. These observations indicate that a reintroduction program should utilize not only exclusively Virginia stocks, but should restrict transfers to the level of watershed. This 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. Thus, there is an important balance to be struck within a restoration-based breeding program. First, to avoid deleterious population mixing effects (i.e., outbreeding depression) we caution against transfer of individuals or gametes across watersheds or regions. Second, to avoid inbreeding effects, we urge a breeding program that incorporates pedigrees or other means of assessing the potential for inbreeding so as to avoid increased levels of genetic drift within a watershed. 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 examine nuclear gene variability.

Genetic data such as these are necessary for developing an appropriate management plan and research strategy for *E. chaetodon* in Virginia. For example, both the Virginia Blackbanded Sunfish Recovery Plan (Smogor et al. 1995) 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. In addition to documenting the prior distribution of the species and the quality of existing habitat as done by Smith et al. (2000), consideration of genetic data is imperative prior, during, and following any reintroduction activity. 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.

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