Feasibility of using molecular tags for use in the Roanoke River American shad hatchery program

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Summary of major findings

1) **Twelve microsatellite markers provide enough information to successfully match returning American shad back to their respective 2009 parent pair**
2) **Of the observed 39 female and 35 males allowed to tank spawn, only 4 females and 15 males were found to actually produce the progeny that were sampled**
3) **The reduction in actual spawning individuals severely reduced the genetic diversity and effective population size in the offspring group**

Purpose

The Warms Springs Conservation Genetics Lab (CGL) was asked to provide general population genetic parameters and perform parentage analysis on the 2009 Roanoke River, American shad, brood stock and their progeny. This was done to evaluate the utility of molecular tags for marking juvenile Atlantic shad in the Roanoke River, NC and provide preliminary assessments and recommendation to the hatchery program.

Introduction

For restorations to be successful, the source population should have a high degree of genetic diversity, and genetic and environment similarity to that of the new or recipient population (Miller and Kapuscinski 2003). Unfortunately, little is known about the genetic diversity of this Roanoke River American shad population and those used as brood stock. Herein, we use microsatellite DNA data to generate preliminary estimates of genetic diversity for American shad in the Roanoke River, compare indices of genetic diversity between brood and progeny, and perform parentage analysis to estimate the actual number of spawners contributing to a tank spawn. Finally we estimate effective population size and compare this value to that of expected and provide guidance for future North Carolina American shad conservation programs.

Methods

CGL was provided fin clips from a portion of the 2009 adult brood stock of American shad (n = 59) and a representative sample of their progeny (n = 96). DNA was extracted from ethanol preserved fin clips using the DNeasy® Blood and Tissue kit[1] (QIAGEN, Inc., Valencia, California) protocol.

We used multiplex PCR reactions to amplify a suite of 14 microsatellite markers known to amplify in American shad (Julian and Bartron 2007). Multiplex PCR amplifications (Table 1) were performed in 20 µL reactions using the following reaction components: 1× *Taq* reaction buffer (Applied Biosystems Inc., Foster City, California), 3.75 mM $MgCl_2$, 0.423 mM of each dNTP, 0.25 µM of each primer, and 0.08 U *Taq* polymerase (Applied Biosystems, Inc.). PCR conditions were an initial denaturation at 94 ºC (10 min), followed by a touchdown procedure involving 33 cycles and consisting of denaturing (94 ºC), annealing, and extension (74 ºC) cycles, where the initial annealing temperature was initiated at 56 ºC and decreased by 0.2 ºC/cycle.

Prior to electrophoresis, 2 µL of a 1:100 dilution of PCR product was mixed with a 8 µL solution containing 97% formamide and 3% Genescan™ LIZ® 500 size standard (Applied Biosystems, Inc.). Microsatellite reactions were visualized with an ABI 3130 genetic analyzer (Applied Biosystems, Inc.) using fluorescently labeled forward primers and analyzed using GeneMapper® software v3.7 (Applied Biosystems, Inc.). The resulting genotypes were assessed for scoring errors attributable to stutter-products, large allele drop-out, or null alleles, and were tested for conformance to per locus Hardy-Weinberg equilibrium (HWE) using MICRO-CHECKER v2.2 (van Oosterhout et al. 2004). Tests for gametic

[1] Use of tradenames throughout the manuscript does not constitute endorsement by the United States government.

disequilibrium (all pairs of loci in each sample) and a global test of population (cohort) differentiation (10,000 randomized data sets) were performed using FSTAT v2.9.3 (Goudet 2001). All *P*-values were adjusted for multiple comparisons using a sequential Bonferroni method (Rice 1989). A *t*-test (one tail; Sokal and Rohlf 1995) was used to test for homogeneity in allele number, allelic richness, and gene diversity between the 2009 brood stock and a sample of their offspring. Normality and equality of variance for these parameters were assessed with the Kolmogorov-Smirnov and *F*-tests, respectively (Sokal and Rohlf 1995)

We used the computer program PAPA (Duchesne et al. 2002) to estimate the number of loci necessary for 100% allocation success (no genotyping error rate was assigned) and perform parentage analysis. As a precaution, we ran parentage analyses without sex information. After analyses, we then made checked that all crosses were between a male and a female. Parentage analysis was also conducted using CERVUS v3.0 (Kalinowski et al. 2007).

We estimated and compared observed and expected effective population size based on parentage analysis and known demographic information, respectively as defined by

$$N_e = \frac{4(N_{male})(N_{female})}{(N_{male}) + (N_{female})}$$

(Wright 1931); where N_{male} and N_{female} were the number of males and females used in the spawning tank. We compared this value to that of observed based on parentage analyses.

Results

A total of 56 of 59 adult and 96 of 96 juvenile American shad were successfully analyzed using 14 microsatellite markers (Table 1). All loci conformed to per locus (all $P > 0.07$) and global ($P = 0.063$) HWE except one locus (*D21*). Microsatellite marker *D21* had a general excess of homozygotes for most allele size classes, suggesting the existence of null alleles. This locus removed from all subsequent analyses. Average observed number of alleles and heterozygosity were 15.45 and 0.83 (Table 1) which suggest a rather genetically diverse population. There was a significant ($P = 0.027$) difference between parent and offspring samples indicating that the brood stock or progeny contains several genetically distinct units. Significant (10 of 78 comparisons significant after sequential Bonferroni correction) departures in gametic disequilibrium pertaining to the juvenile (but not brood stock) samples suggested that this group may be comprised of multiple genetically distinct units; conversely, this may be an artifact of a loss in genetic diversity between brood and progeny.

Five (*D30, D429, D312, C249*, and *D42*) of twelve loci were necessary to yield 100% allocation success between progeny and parents assuming that no genotyping error occurred. Results of parentage analyses using either PAPA or CERVUS indicated that only 37% of the juveniles successfully assigned to a paired mating (TABLE 2). The observed allocation success rate suggests that that not all brood were represented in the sample.

Parentage analyses indicated that of the 39 female and 35 males allowed to tank spawn, only 4 females and 15 males were found to actually produce the progeny that were sampled. The expected effective population size based on 39 females and 35 males was 73 (using above equation); whereas the observed effective size was 8 (using the same equation). This large discrepancy was alarming and should be monitored to see if it is consistent among tanks, as well as, taken into account in the breeding program for this population (see below).

After subsequent removal of non allocated juveniles, we reexamined the data for differences in genetic diversity between brood stock and progeny. We observed significant differences in genic differentiation between groups (P = 0.001), and a test for gametic disequilibrium in the juveniles was still significant indicating that genetic drift-generated disequilibrium may be occurring in the juvenile samples. Indeed, the number of alleles and observed heterozygosity estimates (two measures of genetic diversity) in the juveniles were significantly (P = 0.0004 and 0.01; respectively) less than that of the brood stock.

Discussion

Feasibility of molecular tags

Conventional tags for marking are prone to failure because of battery life, tissue regeneration, or loss of external/internal tag. As such, they often give unreliable estimate of census numbers when used in mark-recapture studies or to assess hatchery contributions. In contrast, molecular tags do not suffer from these inadequacies and are a part of the fish until death. However, a genetic problem can preclude individual identification. If not enough loci are examined, multilocus genotypes in a population may not be unique. If this happens, individuals with the same genotype will be indistinguishable.

This problem can be remedied by having enough loci to minimize the probability that two individuals have the same multilocus genotype. This probability of identity is a standard statistic in forensic science to evaluate how well a set of molecular markers discriminates between individuals and can be evaluated from allele frequencies in a population using established formulae. We found that five microsatellite markers were sufficient to discriminate progeny from 59 contributing brood stock. Simulations based on the allele frequencies of the brood stock indicated that twelve loci can be used to perform parentage analyses on brood stocks of at least 3000 individuals (simulations were stopped at this point due to time constraints). These findings indicate that these markers when used as a molecular tag provide a feasible alternative to conventional tagging methods. A cost break down of such analyses is provided in the Appendix 1.

Loss of genetic variation in brood stock

Loss of genetic diversity reduces adaptive capacity and increases the risk of extinction (Franklin 1977); therefore, a primary goal for conservation hatchery programs is to maintain similar genetic resources and life history patterns between hatchery and natural populations (Lynch and O'Hely 2001, Wedekind 2002). Founding or augmenting a population using a small number of individuals (the founder effect) can cause a loss of genetic variation; however the predicted loss is expected to be

different depending on the measure of genetic diversity (Allendorf 1986). Rare alleles are predicted to be especially susceptible to loss; in contrast, heterozygosity should remain relatively unaffected (Allendorf, 1986). The microsatellite data presented here showed that 29% (58 of the 199) of the alleles observed in the brood stock were not sampled in juveniles (i.e., the allocated juveniles). The observed heterozygosity was also significantly less than that of the brood stock which is alarming because heterozygosity is much more insensitive to a founder effect.

The subsequent loss of genetic diversity is also captured in the stark contrast between observed and expected estimates of effective population size. Effective population size places an upper limit on the amount of genetic diversity that can be maintained in a population or species relative to its pedigree history and potential future losses due to genetic drift. Some general guidelines for maintaining minimum N_e in distinct populations have been proposed:

- N_e > 50 to prevent inbreeding depression and a detectable decrease in viability or reproductive fitness of individuals in a population (Franklin 1980).
- N_e > 500 to maintain constant genetic variance in a population resulting from a balance between loss of variance due to genetic drift and the increase in variance due to spontaneous mutations (Franklin 1980 (Franklin 1980, Lande 1988, Soule 1980);
- N_e > 5,000 to maintain a constant variance for quasi-neutral genetic variation that can serve as a reservoir for future adaptations in response to natural selection and changing environmental conditions (Lande 1995). The rationale here is that N_e should be large enough to minimize genetic drift and the potential loss of "neutral" alleles that may confer fitness advantages under changing environmental conditions and new selection regimes.

The N_e > 500-5,000 rule applies primarily to an entire ESU or species over evolutionary time scales, whereas N_e > 50 applies primarily to closed, local populations over very short time scales (2-5 generations). Thus while the expected N_e (73) was an appropriate target for the guidelines listed above, the observed value was much less than that of expected indicating the potential for a severe loss in genetic diversity of American shad during tank spawning. This loss, if not taken in to account (see below), could actually impede rather than help conservation recovery of the population.

Restoration and management recommendations.

The microsatellite data presented here showed that selecting a limited number of fish for brood was sufficiently small to cause loss of alleles at presumably neutral genes. While such alleles may not contribute significantly to extant quantitative genetic variation, they may be crucial for the long-term adaptive potential of a population under changing environmental conditions. Steps in the hatchery can be implemented to minimize further loss of genetic diversity; thereby, increasing the likelihood of successful reintroductions.

Hatchery managers can protect the genetic viability of the hatchery portion of a population in the short-term by keeping N_e of the brood stock spawned in the hatchery each year above a threshold

target value of at least 50 (Franklin 1980). The target value can be estimated from the numbers of males and females used as brood stock to produce that hatchery group. For example, if 25 male-female pairs are spawned in a hatchery, and if variance in family sizes is random through all stages of the life cycle of their offspring, then N_e of that cohort of offspring would equal the census size, N = 50. Any factor that causes selection among family groups can increase the variance in family size and subsequently reduce N_e. These factors can include (but are not limited to), artificial selection, size-selected predation, isolated disease outbreaks, and varying environmental conditions – none of which are mutually exclusive. Many of these factors are uncontrollable in the hatchery but by equalizing the variance in family size at an early life stage and maintaining an equal sex ratio in the hatchery, some of these factors can be controlled for as a means to maintain higher N_e (Allendorf 1993).

Often conservation hatcheries are pressed to find adequate numbers of brood stock to fulfill the minimum N_e because of the depressed census size of many threatened or endangered species or because very little is known about the captive propagation of such species. This problem can be overcome by recognizing that N_e is a per generation value where the generation interval is the average age of reproduction (Harris and Allendorf 1989). If the average age of reproduction is known, one can simply divide N_e by the generation interval (Waples 1990) to assess the number of brood stock necessary each year for spawning and then randomly spawn this number (1:1 mating pairs) for the duration of the generation interval (relatedness results of our study indicate that random spawning is sufficient to avoid inbreeding in the mating scheme). If paired matings are not possible or undesirable (Fiumera et al. 2004) or if less than the projected brood can be obtained, the conservation plan can simply extend the propagation program past the generation interval until the desired N_e is realized. Extending the propagation plan also allows for the maintenance of rare alleles. The success of a conservation hatchery plan must, therefore, be dependent on a firm commitment by state and/or federal agencies to support, maintain, and monitor the reintroduction program over the entire generation of the organism.

Summary

Knowledge of a species genetic diversity should be incorporated into management/conservation plans prior to hatchery augmentation or reintroduction. In doing so, it provides baseline data to monitor and quantitatively evaluate success of such programs. Our data indicated that the genetic diversity of the brood stock remains relatively high. This observation appears to be the result of the presumed large census size of American shad in the Roanoke River. We also found significant differences in the average number of alleles (and observed heterozygosity) between the hatchery brood stock and their progeny suggesting that the genetic diversity of the sampled juveniles was reduced. The reduction can be attributed to sampling few numbers of individuals contributing offspring to the next generation due to the tank spawning process. This situation can be remedied by using more individuals as brood stock each year (but <50% of the wild population) or by extending propagation activities over the generation interval. Regardless, populations established or augmented by hatchery stockings should be closely monitored for success.

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Table 1. Microsatellite loci used for the estimation of genetic parameters in sampled American shad. The abbreviations are as follows: *k,* number of alleles; *N,* sample size; and H_{obs} and H_{exp} are observed and expected heterozygosity values (asterisk represents significant departures from Hardy Weinberg equilibrium). Superscript letters represent loci run under the same PCR multiplex.

Locus	*k*	*N*	H_{Obs}	H_{Exp}
D30[a]	20	138	0.891	0.916
D31[a]	12	141	0.837	0.836
D429[a]	10	140	0.857	0.863
D21[a]	12	137	0.737	0.824*
B20[b]	11	136	0.787	0.809
C59[b]	17	134	0.799	0.852
D312[b]	15	136	0.816	0.872
D55[b]	13	135	0.763	0.73
C249[c]	22	145	0.883	0.888
C334[c]	20	146	0.856	0.878
D42[c]	17	147	0.939	0.92
D392[d]	20	142	0.782	0.851
D29[d]	12	136	0.809	0.875

Table 2. Results of American shad parentage analysis. Offspring and candidate parent ID corresponds to USFWS numbers (see Appendix 2 for correspondence to NC tag numbers). An asterisk in the Trio confidence column indicates strict confidence for the most likely candidate parent pair (a blank in this column indicates no parent pair could be confidently assigned to an offspring candidate).

Offspring ID	Loci typed	First candidate ID	Sex candidate 1	Second candidate ID	Sex candidate 2	Trio confidence
11917	14	12340	m	12366	f	*
11928	14	12370	m	12390	f	*
11929	14	12347	f	12379	m	*
11930	14	12347	f	12348	m	*
11935	14	12347	f	12379	m	*
11940	14	12361	f	12381	m	*
11941	10	12358	m	12390	f	*
11947	14	12341	m	12361	f	*
11961	10	12356	m	12390	f	*
11962	14	12379	m	12390	f	*
11966	14	12347	f	12356	m	*
11971	10	12340	m	12366	f	*
11972	14	12340	m	12366	f	*
11983	13	12347	f	12350	m	*
11987	10	12361	f	12370	m	*
11993	10	12349	m	12390	f	*
11994	14	12340	m	12366	f	*
11995	14	12347	f	12360	m	*
11996	13	12347	f	12382	m	*
11998	13	12340	m	12366	f	*
12001	13	12350	m	12390	f	*
12006	14	12366	f	12370	m	*
12007	14	12340	m	12366	f	*
11918	14	12361	f	12364	m	*
11925	14	12361	f	12377	m	*
11974	14	12335	m	12390	f	*
11981	14	12347	f	12379	m	*
11982	14	12358	m	12390	f	*
11984	14	12379	m	12390	f	*
11986	10	12366	f	12382	m	*
11970	14	12347	f	12370	m	*
11988	14	12347	f	12350	m	*
12008	10	12340		12356		
11951	12	12341		12366		
11954	10	12355		12356		
11963	13	12350		12361		
11991	14	12339		12370		

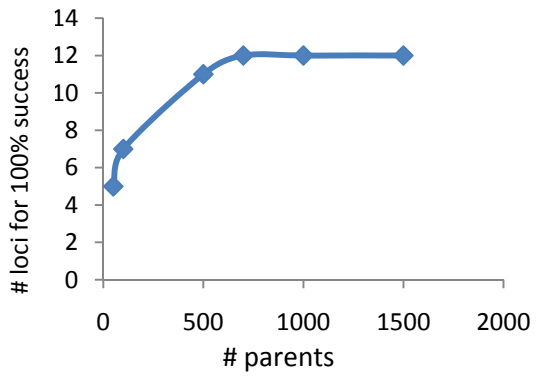
12002	12	12352	12356
11933	14	12339	12366
11936	13	12350	12391
11939	10	12356	12360
11944	14	12341	12388
11946	14	12356	12376
11956	13	12350	12353
11964	13	12356	12359
11977	14	12356	12366
11978	14	12339	12356
11980	14	12377	12379
11990	14	12351	12356
11992	14	12339	12377
11999	14	12356	12359
12011	12	12339	12342
11926	12	12356	12376
11927	14	12378	12382
11931	13	12356	12384
11943	14	12339	12388
11945	12	12355	12356
11948	14	12339	12340
11949	14	12356	12359
11950	14	12339	12341
11952	14	12353	12356
11957	14	12341	12356
11959	13	12341	12386
11965	14	12356	12370
11968	14	12341	12356
11979	14	12339	12386
11997	14	12364	12366
12000	13	12341	12369
12004	13	12356	12375
12005	14	12337	12339
11919	14	12351	12356
11920	14	12341	12384
11934	13	12343	12344
11942	14	12343	12382
11960	14	12366	12386
11973	14	12356	12383
11975	14	12339	12370
11976	14	12356	12386
11989	14	12342	12350
12010	13	12337	12358

11924	14	12380	12386
11958	14	12366	12381
11967	14	12369	12372
12009	14	12339	12369
11932	14	12336	12376

Appendix 1. Cost of molecular tags for use in Roanoke River American shad hatchery programs

The price of using molecular tags is contingent on the number of brood stock and number of returning progeny; therefore, it is difficult to provide actual costs without a firm understanding of these numbers. Below is an attempt to quantify some of the associated costs in such a way that a hatchery manager or field biologist can use if the number of brood and returns is known or estimated.

As the number of brood stock increases so does the number of markers necessary for 100% allocation success. Figure 1 is an illustration of this and is based on the allele frequencies of the 2009 brood stock used in this study. Thus for 500 brood stock, it would be necessary to use 11 microsatellite markers as the molecular tag to achieve 100% allocation success



Below is an example for estimating project costs based on current laboratory cost for CGL (these prices are subjected to increases based on vendor pricing but are current for 2010). The hatchery manager or field biologist can simply adjust the number of samples and markers accordingly. Note that salary of an employee guarantees rapid results, but is not necessary.

Lab protocol	No. Samples	Price/Sample	No. markers	Total
DNA Extraction:	100	$3.24	12	$324.00
PCR:	100	$1.85	12	$2,220.00
Genotyping:	100	$2.11	12	$2,532.00
Fixed cost:	Capillary (1)			$898.00
	10x buffer (1)*			$95.97
ABI service contract (25% of chemical costs)				$1,517.49
Travel				$500.00
1/4 FTE (GS 9) salary***				$10,393.00
Benefits				$4,157.00
Overhead (17%)				$3,848.37
			Overall total*	***$26,485.83***

* increase quantity by one, every 300 samples

** based on 100 samples and 12 microsatellite markers

*** guarantees rapid (~1-3months) turnaround time of samples

Appendix 2. USFWS and North Carolina tagging information for the 2009 brood stock and juvenile samples of America shad collected in the Roanoke River.

USFWS	NC	USFWS	NC	USFWS	NC	USFWS	NC
11820	101	**11885**	166	**11950**	231	**12015**	296
11821	102	**11886**	167	**11951**	232	**12016**	297
11822	103	**11887**	168	**11952**	233	**12017**	298
11823	104	**11888**	169	**11953**	234	**12018**	299
11824	105	**11889**	170	**11954**	235	**12019**	300
11825	106	**11890**	171	**11955**	236	**12334**	301
11826	107	**11891**	172	**11956**	237	**12335**	302
11827	108	**11892**	173	**11957**	238	**12336**	303
11828	109	**11893**	174	**11958**	239	**12337**	304
11829	110	**11894**	175	**11959**	240	**12338**	305
11830	111	**11895**	176	**11960**	241	**12339**	306
11831	112	**11896**	177	**11961**	242	**12340**	307
11832	113	**11897**	178	**11962**	243	**12341**	308
11833	114	**11898**	179	**11963**	244	**12342**	309
11834	115	**11899**	180	**11964**	245	**12343**	310
11835	116	**11900**	181	**11965**	246	**12344**	311
11836	117	**11901**	182	**11966**	247	**12345**	312
11837	118	**11902**	183	**11967**	248	**12346**	313
11838	119	**11903**	184	**11968**	249	**12347**	314
11839	120	**11904**	185	**11969**	250	**12348**	315
11840	121	**11905**	186	**11970**	251	**12349**	316
11841	122	**11906**	187	**11971**	252	**12350**	317
11842	123	**11907**	188	**11972**	253	**12351**	318
11843	124	**11908**	189	**11973**	254	**12352**	319
11844	125	**11909**	190	**11974**	255	**12353**	320
11845	126	**11910**	191	**11975**	256	**12354**	321
11846	127	**11911**	192	**11976**	257	**12355**	322
11847	128	**11912**	193	**11977**	258	**12356**	323
11848	129	**11913**	194	**11978**	259	**12357**	324
11849	130	**11914**	195	**11979**	260	**12358**	325
11850	131	**11915**	196	**11980**	261	**12359**	326
11851	132	**11916**	197	**11981**	262	**12360**	327
11852	133	**11917**	198	**11982**	263	**12361**	328
11853	134	**11918**	199	**11983**	264	**12362**	329
11854	135	**11919**	200	**11984**	265	**12363**	330
11855	136	**11920**	201	**11985**	266	**12364**	331
11856	137	**11921**	202	**11986**	267	**12365**	332
11857	138	**11922**	203	**11987**	268	**12366**	333
11858	139	**11923**	204	**11988**	269	**12367**	334
11859	140	**11924**	205	**11989**	270	**12368**	335
11860	141	**11925**	206	**11990**	271	**12369**	336
11861	142	**11926**	207	**11991**	272	**12370**	337
11862	143	**11927**	208	**11992**	273	**12371**	338
11863	144	**11928**	209	**11993**	274	**12372**	339
11864	145	**11929**	210	**11994**	275	**12373**	340
11865	146	**11930**	211	**11995**	276	**12374**	341

11866	147	**11931**	212	**11996**	277	**12375**	342
11867	148	**11932**	213	**11997**	278	**12376**	343
11868	149	**11933**	214	**11998**	279	**12377**	344
11869	150	**11934**	215	**11999**	280	**12378**	345
11870	151	**11935**	216	**12000**	281	**12379**	346
11871	152	**11936**	217	**12001**	282	**12380**	347
11872	153	**11937**	218	**12002**	283	**12381**	348
11873	154	**11938**	219	**12003**	284	**12382**	350
11874	155	**11939**	220	**12004**	285	**12383**	351
11875	156	**11940**	221	**12005**	286	**12384**	352
11876	157	**11941**	222	**12006**	287	**12385**	353
11877	158	**11942**	223	**12007**	288	**12386**	354
11878	159	**11943**	224	**12008**	289	**12387**	355
11879	160	**11944**	225	**12009**	290	**12388**	356
11880	161	**11945**	226	**12010**	291	**12389**	357
11881	162	**11946**	227	**12011**	292	**12390**	358
11882	163	**11947**	228	**12012**	293	**12391**	359
11883	164	**11948**	229	**12013**	294	**12392**	360