Rare mussel conservation genetics:

Appalachian Elktoe and Brook Floater in western NC

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Date: 25 May 2010

Appalachian Elktoe (*Alasmidonta raveneliana*) and Brook Floater (*A. varicosa*) populations in North Carolina are small, fragmented, and vulnerable to chance events that may periodically reduce population sizes further. Floods in 2004 reduced populations of both species (Fraley and Simmons, 2006). Appalachian Elktoe have experienced unexplained die-offs recently in the Little Tennessee River (NCWRC, unpublished data 2006). Over time, these stochastic events can lead to losses of genetic variation due to non-selective forces, such as inbreeding and genetic drift. Population genetic data from all extant populations can help direct management of wild populations, captive breeding programs, and augmentation and reintroduction efforts to conserve genetic variation within and among populations, while conserving sustainable demographics and populations. Nothing is presently known about the inter- and intra-population genetics of the Appalachian Elktoe and Brook Floater.

The NCWRC, NCSM and NCSU, with support from NCDOT, USFWS, and others, are partnering to develop, refine, and implement captive propagation technology for freshwater mussels. Progeny from captive propagation and translocation of wild adults may allow augmentation of recruitment and effective population size in small, isolated populations, and restoration in streams within the species' historical range. Genetic considerations are an important part of increasing the chances for long-term success of mussel augmentation and reintroductions (National Native Mussel Conservation Committee 1998; Jones et al 2006).

Augmentation of Appalachian Elktoe is an objective of the cooperative Cheoah River restoration effort. Restoration of Brook Floater at suitable localities in the upper Catawba River system is an intermediate goal and augmentation in the Linville River is under consideration by NCWRC. To better categorize appropriate efforts for directing these various augmentation/reintroduction/restoration efforts, a baseline dataset must be assembled that adequately describes population structure of these extant populations. A pilot study found that primers developed for thirteen DNA microsatellite loci in the Dwarf Wedgemussel, *Alasmidonta heterodon*, genome were not capable of resolving useful information from these two species (Tim King, USGS-BRD, pers. comm. 2007). Here, I report results from laboratory efforts towards the development of appropriate primers for microsatellite DNA analysis as well as more routinely reported sequence data (COI, ND1) from minimally invasive sampling of DNA extracted from tissue samples. Data are used to compare among populations sampled and to make management recommendations for recovery of these species.

Methods

Tissue samples (small clips of mantle tissue, or clips from salvaged natural mortalities) of *Alasmidonta varicosa* and *A. raveneliana* were collected from sites listed in Tables 1 & 2 using minimally invasive procedures when necessary. Samples were preserved in 95% ethanol in the field and stored until transported to the NC State Museum of Natural Sciences (NCSM). Whole salvaged individuals were incorporated into the museum's collections and assigned catalog numbers following institutional procedures. Tissues not

associated with museum vouchers were designated unique identifiers based upon the locality from which they were sampled. Total cellular DNA was extracted from all samples using the ArchivePure DNA Tissue kit by 5 Prime (formerly PureGene by Gentra Systems) following the manufacturer's recommendations. Integrity of the DNA was assessed visually on 1-2% agarose gels. DNA concentrations were assessed using a NanoDrop spectrophotometer and diluted to a laboratory standard of 20-50 ng/µL, when required.

Table 1. Sampling localities for *Alasmidonta varicosa* included for this report:

Sample name	Number	Locality	Field number	System
JRAV	6	NC: Burke Co., Johns R.	0509002.1sjf	Catawba, Johns
WCAV	13	NC: Caldwell Co., Wilson Cr.	050901.2sjf & 050901.3sjf	Catawba, Johns
UCAV	19	NC: Burke Co., Upper Cr.	050829.1sjf	Catawba, Warrior Fork
LRAV	6	NC: Burke Co., Linville R.	051003.1sjf	Catawba, Linville
NCSM 43966	1	PN: Tioga Co., Pine Cr.	INVERT09588	Chesapeake, Susquehanna

Table 2. Sampling localities for *Alasmidonta raveneneliana* included for this report:

Sample name	No. of Indiv's	Locality	Field number	System
TRAR	19	NC: Jackson Co., Tuckasegee R.	051013.1sjf & 051013.2sjf	Little Tennessee
CHEO	8	NC: Graham Co., Cheoah R.	070525.1sjf	Little Tennessee
NCSM 27755	1	NC: Swain Co., Little Tennessee R.	INVERT06106	Little Tennessee
NCSM 29606	5	NC: Macon Co., Little Tennessee R.	040407.1sjf	Little Tennessee
NCSM 29746	9	NC: Macon Co., Little Tennessee R.	040423.1sjf	Little Tennessee
NCSM 30861	2	NC: Macon Co., Little Tennessee R.	INVERT06871	Little Tennessee
NCSM 35105	3	NC: Swain Co., Little Tennessee R.	051114.1jws	Little Tennessee
NCSM 40285	1	NC: Swain Co., Little Tennessee R.	051012.1jws	Little Tennessee
LR(FB)AR	19	NC: Transylvania Co., Little R.	050915.1sjf	French Broad
PRAR	16	NC: Haywood Co., Pigeon R.	050927.3jws & 050927.3jws	French Broad
NOLI	16	TN: Unicoi Co., Nolichucky R.	070822.	French Broad
CRAR	5	NC: Yancey Co., Cane R.	051005.2sjf	French Broad, Nolichucky
NTAR	20	NC: Mitchell-Yancey Co., North Toe R.	051004.1sjf & 051004.2sjf	French Broad, Nolichucky
STAR	20	NC: Yancey Co., South Toe R.	050922.2sjf	French Broad, Nolichucky, North Toe
27215	2	NC: Mitchell-Yancey Co., North Toe R.	INVERT05759	French Broad, Nolichucky
40284	1	NC: Mitchell-Yancey Co., North Toe R.	051004.2sjf	French Broad, Nolichucky
Araven(noli)	1	TN: Unicoi Co., Nolichucky R.	uncatalogued	French Broad

Mitochondrial analysis

PCR primers (Folmer et al., 1994; Serb et al., 2003) and conditions are shown in Table 3. DNA sequencing was performed using the same primers used for PCR following the recommended protocol with the ABI BigDye v. 3.1 sequencing kit (Applied Biosystems). Sequences were purified for sequencing using the Qiagen DyeEx Spin Kit (Qiagen) or utilizing ABI's recommended protocol and visualized using either an ABI 377 or ABI 3130 XL automated sequencer (Applied Biosystems). Sequences presented in this report were generated *de novo*. All sequence data presented herein represent a part of a much larger effort examining relationships among *Alasmidonta* species. Because of this, only information relevant to the project at hand will be reported herein pending publication of the larger study.

Sequence data was compiled using Sequencher™4. Alignments were assembled into a NEXUS file for phylogenetic analysis. A preliminary bootstrap analysis was conducted using Seaview 4.2 (Gouy et al., 2010) with ND1 and COI data combined into a single continuous data matrix. More comprehensive analyses await completion of our *Alasmidonta* dataset, but sufficient inferences may be drawn from the data presented here.

Table 3. Primers used in the phylogenetic analysis.

Name		Sequence	Amplification
ND1	Leu-uurF	5'-TGGCAGAAAGTGCATCAGATTAAAGC-3'	30cycles: 94 °C, 1min; 52 °C, 1min; 72 °C, 2min
	LoGlyR	5'-CCTGCTTGGAAGGCAAGTGTACT-3'	
COI	COI-H	5'-TAAACTTCAGGGTGACCAAAAAATCA-3'	35cycles:94 °C,30s; 54 °C, 30s;72 °C, 1min30s
	COI-L	5'-GGTCAACAAATCATAAAGATATTGG-3'	

Microsatellite development

One DNA sample each from *A. raveneliana* and *A. varicosa* was sent to the Savannah River Ecology Laboratory's (SREL) DNA lab for development of microsatellite enriched libraries for a fee. Libraries were returned to the lab (NCSM) for further processing following SREL's recommended protocol (Glenn & Schable, 2005). From these libraries more than 1,000 sequences were generated in order to search for and develop microsatellite loci for analyses. Sequences were compiled in Sequencher™4 and identical clone sequences were combined. To date, more than 300 of these sequences have been analyzed for primer development using the program msatcommander (Faircloth, 2008) to characterize repeats and to design "tagged" primers for PCR amplification and microsatellite analysis. As of the writing of this report, 26 primer pairs have been purchased and 8 of these have been used successfully for the data reported herein. The remaining 18 potential loci are currently untested pending a need for utilization of additional loci.

Primers & summary statistics for microsatellite loci are listed in Table 4. Primers were designed using a scheme to provide for maximum flexibility in fluorescent labeling following a modification of Schuelke (2000) utilizing a "CAG" tag (CAGTCGGGCGTCATCA with bases common to the microsatellite primer removed) instead of the M13(-21) cited in that publication. The 15 µL PCR reaction mixture consisted of either a FAM- or a HEX-labeled fluorescent tag (primer) with a limiting concentration of CAG-labeled forward primer as follows: 8.0 pmol of reverse primer, 8.0 pmol of one of the two fluorescent tags, 2 pmol of CAG-labeled forward primer, 7.5 µL of Promega GoTaq® Colorless Master Mix and approximately 50 ng of genomic template. For each reaction, PCR amplification conditions were as follows: initial denaturation at 94°C for 2 minutes followed by 35 standard PCR cycles of 94°C, 58°C, 72°C each for 30 seconds and a final extension of 72°C for 30 minutes.

Microsatellite PCR products were multiplexed post-PCR (a HEX-labeled reaction product was combined with a FAM-labeled product) and run in the same run/lane on an ABI 3130XL automated DNA sequencer. This allowed for a minimal consumption of lab reagents for the duration of the project. The mixture consisted of 1 µL of each reaction product, 9.25 µL of HiDi™ Formamide and 0.25 µL of Genescan™-500 ROX size standard (both from Applied Biosystems, Inc.) following the manufacturer's recommendation. Data were compiled in GeneMapper 4.0 (Applied Biosystems, Inc.)

The preliminary summary statistics presented in Tables 5-8 were derived using GenAlEx 6.3 (Peakall & Smouse, 2006). Data collection has not yet been completed, so these data are expected to change from what is presented. However, in the case of *A. raveneliana*, I expect no major changes to develop which might affect how these data are interpreted. Additional data should definitely be collected for *A. varicosa* since no robust patterns are currently observed.

Results

Mitochondrial analysis

Mitochondrial samples have been sequenced for both genes, but in several instances, data are missing due to random failures during DNA sequencing. Therefore, the dataset contains several "holes" which will be remedied in the coming months. However, since many phylogenetic algorithms are not robust to missing data, I have presented a simple bootstrap tree (Figure 1) to illustrate the major findings of the mitochondrial analysis. Of note are the following points:

1. Mitochondrial data break *A. raveneliana* into two reciprocally monophyletic units corresponding to the drainage unit from which they originated (Little Tennessee R. versus French Broad R. drainages). In no instance are individuals from one drainage recovered in the clade for the other.
2. Aside from this major break, very limited structure (and likely an artifact of the data) exists within these clades. Additional data (for instance, from microsatellites) are required to tease apart any further population level structure.
3. As with *A. raveneliana*, exceptionally little structure is recovered within/among populations of *A. varicosa* using only mitochondrial sequence data. The only division of note seems to correspond to a cryptic lineage of "*A. varicosa"* which might actually represent the rediscovery of *A. robusta*, a species which was described as a species presumed to be extinct not long after its description in 1981.
4. Catawba populations of *A. varicosa* resolve as a large unresolved polytomy in this analysis. Additional data, both informative genetic loci as well as additional specimens from throughout its range, are required for more comprehensive analysis.

Figure 1. Bootstrap results of mitochondrial analyses for both *A. raveneliana* & *A. varicosa*. Included in this tree are 1,560 bp of sequence data from COI & ND1.

Table 4. Microsatellite primer designed for this project:

Locus	Repeat motif	Primer sequence (5'—3')	Allele size range (bp)	N	A	H_O	H_E
Ara04	$(GT)_{16}$...$(AG)_{26}$...$(G)_{11}$	F: CAGTCGGGCGTCATCAGAGGGCATCCTCTCCAGC	353-397 A. raveneliana	148	23	0.731	0.884
		R: ACAACCAGACCCATACATTCTTG	357-441 A. varicosa	45	19	0.796	0.817
Ara08	$(ATC)_{17}$	F: CAGTCGGGCGTCATCACAGGCATTGACACATGATCG	332-371 A. raveneliana	147	12	0.721	0.668
		R: AGGCTGGCTATACATGGCG					
Ara09	$(GT)_9$...$(GT)_{11}$	F: CAGTCGGGCGTCATCATGCACATTCCTTGAACCGC	215-247 A. raveneliana	136	12	0.657	0.685
		R: GCAAAGATGGGAAGCAGGC					
Ara10	$(GTTT)_6$	F: CAGTCGGGCGTCATCACCCACCCATCCTTCTTCGG	372-392 A. raveneliana	134	4	0.192	0.178
		R: AGTGTTAGGACTGGTCGGC					
Ara17	$(GT)_{14}$	F: CAGTCGGGCGTCATCAGCCTTAGGTGCGCAGAAATAC	288-312 A. raveneliana	137	7	0.792	0.750
		R: GGATCCGACATTTATTTGCCC	286-314 A. varicosa	44	7	1.000	0.738
Ara26	$(GTTT)_6$	F: CAGTCGGGCGTCATCAGGAACAACCCAGTTCCATCG	197-212 A. raveneliana	144	12	0.493	0.588
		R: AGACCACTCGGACATTCGG	187-200 A. varicosa	45	10	0.759	0.758
Ara29	$(AAAC)_5$	F: CAGTCGGGCGTCATCAGACCAGACCAGCACTGGAG	170-186 A. raveneliana	148	2	0.047	0.045
		R: CTGCCTTACAGCAGACATCG	182 A. varicosa	46	1	0	0
Ara30	$(CTGT)_4$	F: CAGTCGGGCGTCATCAAGGGGATCACACGAACCG	208-212 A. raveneliana	147	2	0.116	0.125
		R: ACCCTCTCGCTGTCTTTCTC	208-211 A. varicosa	45	3	0.267	0.418

All primers annealed optimally between 58—60°C with no special changes to the PCR protocol. Forward primers were designed using CAG tags (CAGTCGGGCGTCATCA with common bases removed) as implemented in msatcommander (Faircloth, 2008). N = number of individuals successfully scored, A = number of alleles, H_O = observed heterozygosity, H_E = expected heterozygosity

Microsatellite Development and Analysis

Results for the microsatellite loci tested varied significantly for the two species studied. Eight loci have been effectively amplified for these species. Data from all eight loci are included for this report for *A. raveneliana*. For *A. varicosa*, however, only five loci have currently been scored. Data for the remaining 3 loci still need to be compiled and added to the analysis. Summary statistics are provided for both datasets in tables 5-8.

A. raveneliana results:

In most cases, the majority of the 148 individuals were successfully amplified and scored for the eight loci developed. Deviations from Hardy-Weinberg Equilibrium (HWE) were seen in Ara04, Ara09, Ara10, Ara17, and Ara30, but it is difficult to determine if this phenomenon is real (due to null alleles or biologically significant deviations) or an artifact of small sample sizes that might be overcome by sampling additional individuals, if they are available. Other published datasets have handled this deviation from significance differently. For instance, in Kelly & Rhymer (2005), the authors interpreted their HWE significances to be largely due to the presence of null alleles. Results for Appalachian Elktoe seem likely to contain null alleles, but these analyses have not yet been examined. In a much larger study of 250 Freshwater Pearl Mussels, *Margaritifera margaritifera*, from Europe, deviations were recovered only in populations found to be statistically less diverse (Geist et al., in press). Therefore, both scenarios seem likely possibilities in Appalachian Elktoe. Nonetheless, data for these loci will be reexamined with more statistical analysis prior to submission of a final report and subsequent publication, but for the purposes of this report are not currently interpreted as problematic to the conclusions being presented.

Bayesian population assignment tests using the program Structure v.2.3.2 show high assignment probabilities for these data to accurately identify the two groupings recovered in the mitochondrial data. Figure 2A is a typical result from these data. Populations 1 to 6 are samples taken from the French Broad drainage while populations 7-9 are from the Little Tennessee. Probabilities are high that an individual from these samples, when screened for these microsatellite markers, are correctly assigned back to these 2 populations. However, when the number of populations is increased to 3, as in Fig. 2B, probability of assignment to population 6, the Pigeon R., also becomes high suggesting additional population structure inherent in the dataset. Additional loci should be screened to test this assertion, but there seems to be no evidence of population sub-structure among populations 1-5 (all French Broad populations, except the Pigeon R.) or 7-9 (all Little Tennessee populations). These data seem to support the mitochondrial data in that there are 2 large populations of *A. raveneliana*, one in the French Broad and the other in the Little Tennessee. But these data also support the conclusion that the Pigeon R. sub-population also exhibits some level of identity that should be maintained. Any augmentation efforts are recommended to be restricted to within the large populations (Little Tennessee or French Broad) but data also seem to support not mixing anything exogenous into the Pigeon R. sub-population at this time.

Table 5. Summary statistics for *A. raveneliana* by population for each locus.

Population		ara04	ara08	ara09	ara10	ara17	ara26	ara29	ara30
Little R.	**N**	19	19	17	13	19	19	19	19
	Na	17	10	10	2	7	11	1	2
	Ne	12.448	4.429	7.811	1.166	5.085	4.541	1.000	1.170
	Ho	0.895	0.789	0.824	0.154	0.789	0.842	0.000	0.053
	He	0.920	0.774	0.872	0.142	0.803	0.780	0.000	0.145
	UHe	0.945	0.795	0.898	0.148	0.825	0.801	0.000	0.149
	F	0.027	-0.020	0.056	-0.083	0.017	-0.080	#N/A	0.638
Nolichucky R.	**N**	17	17	16	16	15	17	17	16
	Na	13	7	7	1	10	6	2	2
	Ne	9.797	3.546	4.063	1.000	5.422	2.919	1.061	1.133
	I	2.401	1.509	1.620	0.000	1.963	1.346	0.133	0.234
	Ho	0.588	0.824	0.625	0.000	0.800	0.529	0.059	0.125
	He	0.898	0.718	0.754	0.000	0.816	0.657	0.057	0.117
	UHe	0.925	0.740	0.778	0.000	0.844	0.677	0.059	0.121
	F	0.345	-0.147	0.171	#N/A	0.019	0.195	-0.030	-0.067
Cane R.	**N**	5	5	5	4	5	5	5	5
	Na	8	4	4	1	5	3	1	2
	Ne	7.143	2.778	2.941	1.000	3.125	1.515	1.000	1.724
	Ho	0.600	1.000	0.600	0.000	0.800	0.400	0.000	0.600
	He	0.860	0.640	0.660	0.000	0.680	0.340	0.000	0.420
	UHe	0.956	0.711	0.733	0.000	0.756	0.378	0.000	0.467
	F	0.302	-0.563	0.091	#N/A	-0.176	-0.176	#N/A	-0.429
N. Toe R.	**N**	23	23	22	23	23	23	23	23
	Na	16	9	7	3	7	7	3	2
	Ne	8.891	4.853	3.470	1.249	4.038	2.748	1.092	1.044
	Ho	0.609	0.652	0.682	0.130	0.696	0.565	0.087	0.043
	He	0.888	0.794	0.712	0.199	0.752	0.636	0.084	0.043
	UHe	0.907	0.812	0.728	0.204	0.769	0.650	0.086	0.043
	F	0.314	0.179	0.042	0.346	0.075	0.111	-0.034	-0.022
S. Toe R.	**N**	20	20	19	19	20	20	20	20
	Na	14	9	11	1	10	7	2	2
	Ne	9.877	4.211	4.781	1.000	6.723	2.703	1.105	1.220
	Ho	0.750	0.750	0.737	0.000	0.800	0.500	0.100	0.100
	He	0.899	0.763	0.791	0.000	0.851	0.630	0.095	0.180
	UHe	0.922	0.782	0.812	0.000	0.873	0.646	0.097	0.185
	F	0.166	0.016	0.068	#N/A	0.060	0.206	-0.053	0.444
Pigeon R.	**N**	16	16	16	16	16	16	16	16
	Na	14	6	9	1	5	6	1	2
	Ne	10.240	2.370	4.785	1.000	2.349	2.016	1.000	1.280
	Ho	0.875	0.688	0.750	0.000	0.875	0.438	0.000	0.125
	He	0.902	0.578	0.791	0.000	0.574	0.504	0.000	0.219
	UHe	0.931	0.597	0.817	0.000	0.593	0.520	0.000	0.226
	F	0.030	-0.189	0.052	#N/A	-0.524	0.132	#N/A	0.429
Little Tennessee R.	**N**	21	21	15	16	15	18	21	21
	Na	13	8	8	2	6	2	1	1
	Ne	10.627	3.150	2.459	2.000	3.383	1.906	1.000	1.000
	Ho	0.667	0.619	0.533	0.500	1.000	0.333	0.000	0.000
	He	0.906	0.683	0.593	0.500	0.704	0.475	0.000	0.000
	UHe	0.928	0.699	0.614	0.516	0.729	0.489	0.000	0.000
	F	0.264	0.093	0.101	0.000	-0.420	0.299	#N/A	#N/A

Cheoah R.	**N**	8	7	8	8	7	8	8	8
	Na	6	3	2	2	6	4	2	1
	Ne	4.571	1.556	1.600	1.753	4.083	3.048	1.133	1.000
	Ho	0.750	0.429	0.500	0.625	0.429	0.500	0.125	0.000
	He	0.781	0.357	0.375	0.430	0.755	0.672	0.117	0.000
	UHe	0.833	0.385	0.400	0.458	0.813	0.717	0.125	0.000
	F	0.040	-0.200	-0.333	-0.455	0.432	0.256	-0.067	#N/A
Tuckasegee R.	**N**	19	19	18	19	17	18	19	19
	Na	13	8	6	2	6	5	2	1
	Ne	10.314	3.438	2.592	1.498	5.453	2.464	1.054	1.000
	Ho	0.842	0.737	0.667	0.316	0.941	0.333	0.053	0.000
	He	0.903	0.709	0.614	0.332	0.817	0.594	0.051	0.000
	UHe	0.927	0.728	0.632	0.341	0.841	0.611	0.053	0.000
	F	0.067	-0.039	-0.085	0.050	-0.153	0.439	-0.027	#N/A

Na = No. of Different Alleles

Ne = No. of Effective Alleles

Ho = Observed Heterozygosity

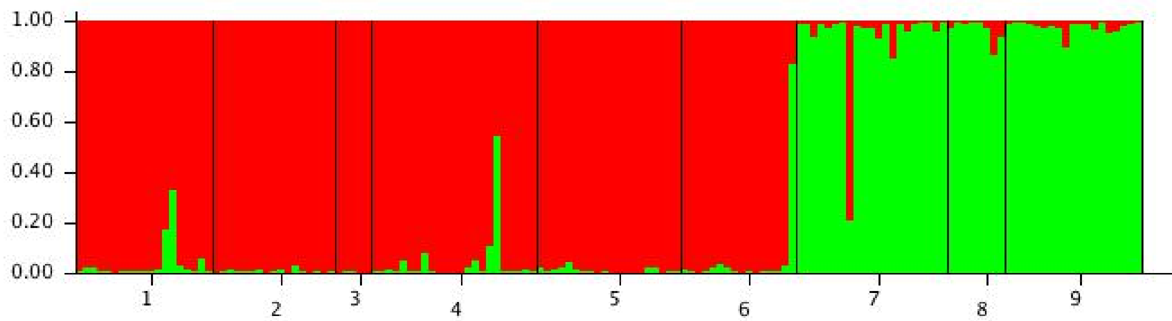
He = Expected Heterozygosity

UHe = Unbiased Expected Heterozygosity

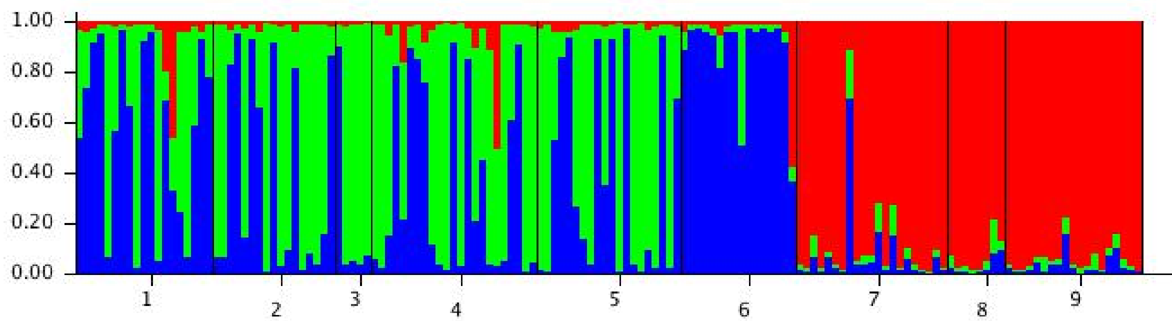
F = Fixation Index

Table 6. Summary F-statistics for *A. raveneliana* for all populations by locus.

	ara04	**ara08**	**ara09**	**ara10**	**ara17**	**ara26**	**ara29**	**ara30**	**Mean**	**SE**
Fis	0.174	-0.078	0.040	-0.076	-0.056	0.160	-0.046	0.069	0.023	0.037
Fit	0.219	0.106	0.177	0.485	0.073	0.360	-0.018	0.177	0.197	0.057
Fst	0.055	0.171	0.143	0.521	0.122	0.237	0.027	0.116	0.174	0.055



A. K=2



B. K=3

Figure 2. Preliminary results of Structure 2.3.2 analysis of the *A. raveneliana* microsatellite dataset. In A, 2 populations are defined correlating to the French Broad R. vs. the Little Tennessee R. populations. In B. 3 populations are assigned. Groupings 1-9 follow the following order: 1. Little R., 2. Nolichucky R., 3. Cane R., 4. North Toe R., 5. South Toe R., 6. Pigeon R., 7. Little Tennessee R., 8. Cheoah R., 9. Tuckasegee R.

A. varicosa results:

At this time, only five of the eight loci screened have been effectively scored. One of these loci, Ara29, is monomorphic at this level of analysis. This eliminates it from any analyses at this time, which leaves only four loci available for this report. In addition, only an exceptionally small sample has been screened to date from a very limited geographic region of this wide-ranging species. Therefore, results at this time are largely inconclusive and speculative at best. Nonetheless, the following results are preliminarily observed.

Deviations from HWE are observed in 3 of the 4 loci: Ara04, Ara26, and Ara30. No simulations have yet been run to check these data for the probability of null alleles but this will be attempted as data collection progresses. Additional samples, either from NCSM's collection or from additional Catawba locales will definitely be required to make conclusions about population-level genetic parameters in these populations. However, it is encouraging that levels of heterozygosity in the four loci examined to date are similar to others reported in the literature.

Bayesian population assignment analysis (Structure) results are shown in Figure 3. Unlike what is recovered with *A. raveneliana,* the loci screened to date are unable to identify any structure among the populations of *A. varicosa* examined. This is likely an artifact of only analyzing four loci, but might prove real once additional data are screened. The results presented for *A. varicosa* in this report are intended only to illustrate the progress made to date on this part of the project, and are not intended for direction making management decisions, at least at this time.

If efforts are begun to augment various populations within the upper Catawba drainages, care should be taken not to accidentally introduce the new/undescribed form of "*A. varicosa*" into these waters. Debate is underway as to whether this form represents a new species or if it actually represents *A. robusta*, a form that was described in 1981 and considered extinct due to the lack of subsequent collection. To further illustrate how much care should be taken in this instance, *A. robusta* was described from a single collection taken from Long Creek in Mecklenburg Co, NC. This creek is a tributary to the lower Catawba, and is the only locality from which *A. robusta* is known. The individuals which are currently showing up in collections due to renewed effort in sampling appropriate habitat which resemble *A. robusta* superficially LACK the character used to define this species. The newer individuals (from which DNA has been isolated and characterized) seem to be collected from the Uwharrie region of NC & SC. However, because *A. robusta* exists as only a type series and as paired valves from which no DNA might be isolated, there remains no genetic basis of comparison for these new individuals to be compared against "good" *A. robusta* until a population or an individual can be collected which also exhibit the morphology (a unique interdental projection) defining this presumed extinct taxon. Given all of this, a possibility remains that if not properly considered, augmentation efforts might accidentally introduce a species native to the system (the Catawba) but not the region (the upper Catawba). Care should be taken to preclude this form of "*A. varicosa"* from any augmentation efforts to the upper Catawba.

Table 7. Summary statistics for *A. varicosa* by population for each locus.

Population		ara04	ara17	ara26	ara29	ara30
NCSM 43966	N	1	1	1	1	1
	Na	2	2	2	1	1
	Ne	2.000	2.000	2.000	1.000	1.000
	Ho	1.000	1.000	1.000	0.000	0.000
	He	0.500	0.500	0.500	0.000	0.000
	UHe	1.000	1.000	1.000	0.000	0.000
	F	-1.000	-1.000	-1.000	#N/A	#N/A
Linville R.	N	6	6	6	6	6
	Na	9	7	7	1	2
	Ne	8.000	6.000	5.143	1.000	1.946
	Ho	0.667	1.000	0.667	0.000	0.167
	He	0.875	0.833	0.806	0.000	0.486
	UHe	0.955	0.909	0.879	0.000	0.530
	F	0.238	-0.200	0.172	#N/A	0.657
Upper Cr.	N	19	19	18	19	19
(Warrior Fork)	Na	15	8	10	1	3
	Ne	10.939	4.658	5.945	1.000	2.242
	Ho	0.789	1.000	0.500	0.000	0.632
	He	0.909	0.785	0.832	0.000	0.554
	UHe	0.933	0.807	0.856	0.000	0.569
	F	0.131	-0.273	0.399	#N/A	-0.140
Wilson Cr.	N	13	12	13	13	12
(Johns)	Na	15	9	11	1	3
	Ne	11.267	5.538	8.244	1.000	2.268
	Ho	0.692	1.000	0.769	0.000	0.250
	He	0.911	0.819	0.879	0.000	0.559
	UHe	0.948	0.855	0.914	0.000	0.583
	F	0.240	-0.220	0.125	#N/A	0.553
Johns R.	N	6	6	7	7	7
	Na	10	6	6	1	2
	Ne	9.000	4.000	4.455	1.000	1.960
	Ho	0.833	1.000	0.857	0.000	0.286
	He	0.889	0.750	0.776	0.000	0.490
	UHe	0.970	0.818	0.835	0.000	0.527
	F	0.062	-0.333	-0.105	#N/A	0.417

Na = No. of Different Alleles

Ne = No. of Effective Alleles

Ho = Observed Heterozygosity

He = Expected Heterozygosity

UHe = Unbiased Expected Heterozygosity

F = Fixation Index

Table 8. Summary F-statistics for *A. varicosa* for all populations by locus.

	Ara04	Ara17	Ara26	Ara29	Ara30	Mean	SE
Fis	0.025	-0.356	0.000	#N/A	0.361	0.008	0.131
Fit	0.145	-0.169	0.134	#N/A	0.591	0.175	0.140
Fst	0.123	0.138	0.135	#N/A	0.360	0.189	0.051

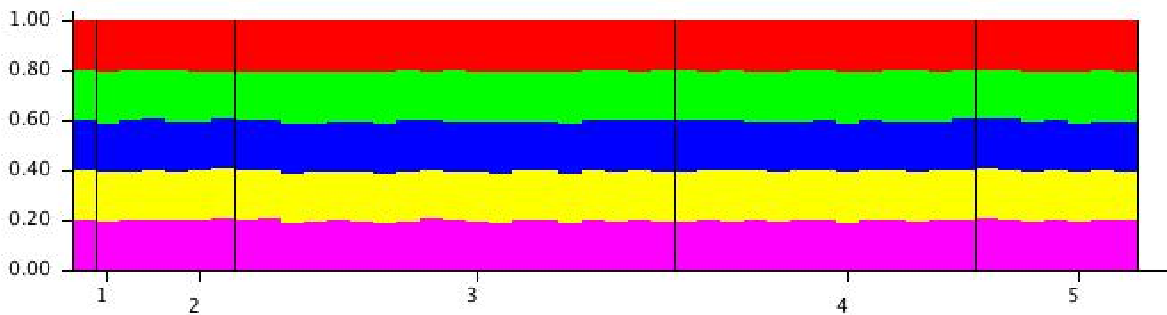


Figure 3. Preliminary results of Structure 2.3.2 analysis of the *A. varicosa* microsatellite dataset. Data from the 5 microsatellite loci collected thus far fail to resolve any differences across these populations. Groupings 1-5 follow the following order: 1. NCSM 43966 Susquehanna R. (Chesapeake), 2. Linville R. 3. Upper Cr. (Warrior Fork) 4. Wilson Cr. (Johns R.), 5. Johns R.

Restoration and management recommendations

A. raveneliana

- Two major populations of Appalachian Elktoe are recovered in both the mitochondrial and nuclear data analyses. These populations should not be mixed if augmentation and restoration are attempted.
- Additionally, individuals from the Pigeon River (French Broad) show a high level of genetic identity. Augmentation efforts should not mix other French Broad populations into this population if at all possible to maintain this diversity.
- Additional data (both more genetic loci as well as a repeated measures sampling efforts) should be collected to better define sup-population structure estimates and to develop more accurate estimates of effective population size (N_e). Repeated sampling might be the only way to accurately estimate N_e in this species especially given the critically small extant populations.

A. varicosa

- Currently, no population or sub-population structure is recovered in either the mitochondrial or nuclear data analyses. The only suggestion of structure seen is the discovery of a previously unrecognized form of Brook Floater, seemingly from the Uwharrie region in the Carolinas.
- Microsatellite loci show promise as good population-level markers for this species, but additional loci and individuals will be required to adequately address the management of the species in the upper Catawba.
- It would not be expected that within the upper Catawba much population level structure should be present, but it is definitely a possibility that should be examined.
- Efforts are underway to increase the number of loci examined for this species. Currently, data are insufficient to make confident recommendations for restoration. Very limited structure among populations throughout the range of the species is possible, but should not be assumed at this time.

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