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Final Report

Genetic Assessment of Two *Stizostedion* Species and Their Hybrid in the Ohio River

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Submitted to Ohio Division of Wildlife

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Genetic Assessment of Two *Stizostedion* Species and Their Hybrid in the Ohio River

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Introduction

Fisheries biologists have begun to evaluate past and current stocking practices and now recognize the importance of genetic stock boundaries for preserving the genetic integrity of natural populations (Kapuscinski and Jacobson, 1987; Meffe, 1987; Kapuscinski and Philipp, 1988; Hindar et al., 1991). Efforts are now concerned with identification and preservation of genetically distinct stocks, so that detrimental effects from stocking on naturally reproducing populations are reduced. Traditionally, this has been a difficult task because the use of morphological analyses alone has likely limited the identification and classification of genetically differentiated populations. With the development of a number of molecular techniques, however, the ability to define distinct stocks has increased. Each molecular technique has the potential to reveal a different amount of genetic variation for each species that is being analyzed, and good comparisons among techniques are now available.

The Ohio River Fisheries Management Team (ORFMT) has identified management of the two *Stizostedion* species and their hybrid (walleye - *S. vitreum*, sauger – *S. canadense*, and saugeye – *S. vitreum* X *S. canadense*) to be a priority in the Ohio River. Concern exists over whether an "Ohio River" form of walleye is still found in the Ohio River and if so, should it be used to stock the river.

This study uses two molecular techniques (allozymes and RFLP analysis of mtDNA) on *Stizostedion* from tailwaters along the Ohio River extending from river mile 54 in Ohio to river mile 918 in Illinois to address two objectives. The first objective using an allozyme analysis was to determine the extent of hybridization between walleye and sauger in any or all of the tailwater areas. The RFLP analysis of mtDNA was used to

determine the direction by which that hybridization was occurring. Because mitochondrial DNA is maternally inherited, an F1 hybrid that had walleye mitochondrial DNA would indicate that male sauger had hybridized with female walleye. An Fx hybrid male (of either direction) that backcrossed with a female walleye would also have walleye mitochondrial DNA. The second objective was to use both allozymes and RFLP analysis of mtDNA in both species to assess genetic variation among populations from tailwater areas on the Ohio River where samples could be collected.

Methods

Collections

Stizostedion samples collected from a total of eight tailwaters from the Ohio River and from Lake Erie were visually identified as walleye, sauger, or hybrid (Table 1). Whole fish were collected and frozen at –20^{O}C until being shipped to the Illinois Natural History Survey in Champaign, Illinois for analysis.

DNA Isolation

Genomic DNA for use in the RFLP analysis was isolated using a technique described by Saghai-Maroof et al. (1984) and modified by Fields et al. (1989). Approximately 200 mg of white skeletal muscle was ground gently in a solution of 100 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1.4 M NaCl, 2% CTAB (Hexadecyltrimethyl-ammonium bromide), and 0.2% 2-mercaptoethanol. Proteinase K was added to a final concentration of 10 µg/mL and the sample was then incubated at 60^{O}C for 35 min. One phenol extraction and two chloroform:isoamyl alcohol (24:1) extractions were used to

remove cellular debris and denatured protein from the DNA. DNA was precipitated overnight at -20°C in 95% ethanol, then centrifuged at 12,000xg, air dried, and resuspended in ddH_2O.

RFLP Analysis of mtDNA

RFLP analysis was performed on two segments (ND-3/4 and Dloop/12s) of the mitochondrial genome that included two genes for each region (NADH subunits 3 and 4) and (Dloop and 12s RNA) to address both objectives. The ND-3/4 mitochondrial genes were amplified using primers by Cronin et al. (1993):

5'-TAA(C/T)TAGTACAG(C/T)TGACTTCAA-3'

5'-TTTTGGTTCCTAAGACCAA(C/T)GGAT-3'

The Dloop/12s RNA mitochondrial genes were amplified using primers by Alvarado Bremer (1994):

5'-AGGGTGACGGGCGGTGTGT-3'

5'-TACCCAAACTCCCAAAGCTA-3'

The polymerase chain reaction mixture modified from Echt et al. (1991) contained the following: approximately 25ng of template DNA, Applied Biosystems 10X Buffer, 2.5 mM $MgCl_2$, 100 µM each of dATP, dCTP, dGTP, and dTTP, 0.08 pmol/µL each of the light and heavy strand primers, and 1.5 units *Taq* polymerase for every 50 µL reaction. Amplification was performed using an MJ Research PTC-100-60 thermocycler with the following profile: an initial denaturation step of 2 min at 95°C, followed by 45 sec at 94°C, 1 min at 45°C, and 2 min 30 sec plus a 4 sec extension/cycle at 70°C. This

protocol was repeated for 29 cycles, followed by a final extension step at 70°C for 4 min plus a final holding step at 4°C.

Subsamples (8µL) of each amplified PCR product were digested with each of nine restriction endonucleases for the ND-3/4 mitochondrial genes (*ALU I*, *BSTU I*, *DDE I*, *DPN II*, *HAE III*, *HHA I*, *HINF I*, *MSP I*, and *RSA I*) and ten restriction endonucleases for the Dloop/12s RNA mitochondrial genes (*SAU3A I* plus the nine listed above) following manufacturer's recommendations. Digests were separated electrophoretically in a gel consisting of 2.0% agarose and 1.0% synergel (Diversified Biotech) in TAE buffer (0.04 M Tris-HCl, 5.7% glacial acetic acid, and 0.001 M EDTA, pH 8.0), with 0.5 µg/mL ethidium bromide to visualize banding patterns under ultraviolet light. Black and white Polaroid photographs were taken of each gel to use in measuring fragments for each banding pattern. A 100 base pair DNA ladder (0.5 µg/lane) was used to determine the size of digested fragments.

Protein Electrophoretic Analysis

Tissue samples of white skeletal muscle, eye, and liver were dissected, homogenized in 100 mM Tris-HCl (pH 7.0), and centrifuged at 22,000xg for 10 minutes at 4°C. Tissue extracts were subjected to vertical starch gel electrophoresis coupled with histochemical staining as described by Philipp et al. (1979) with modifications from Koppelman and Philipp (1986).

Results

RFLP Analysis of mtDNA

Even though poor quality DNA for some individual samples resulted in a lower number of individuals that could be amplified and digested, several amplifications were attempted for each individual (Table 2). Based on the initial screening for variability within both mitochondrial regions, only the Dloop/12s regions were used to analyze all the individuals. A total of ten restriction endonucleases were initially screened for Dloop/12s, however, three of those restriction endonucleases (*ALU I*, *HINF I*, and *SAU 3A I*) could not be resolved and were omitted from further analysis. The remaining seven restriction endonucleases that digested the amplified DNA successfully were *BSTU I*, *DDE I*, *DPN II*, *HAE III*, *HHA I*, *MSP I*, and *RSA I*. Two of the seven restriction endonucleases (*BSTU I* and *MSP I*) gave restriction patterns that were diagnostic between walleye and sauger. Four restriction endonucleases (*DDE I*, *DPN II*, *HAE III*, and *HHA I*) produced polymorphic digestion patterns within walleye individuals and that were diagnostic between walleye and sauger. Lastly, a total of five banding patterns were detected using *RSA I* in walleye and sauger (Table 2). All individuals identified as an F1 or Fx saugeye had one of the same four composite mitochondrial haplotypes as detected in pure walleye (Table 2).

Interestingly, two composite haplotypes were consistently detected using four of the restriction endonucleases (*DDE I*, *DPN II*, *HAE III*, and *HHA I*) in walleye. An individual walleye had either an A banding pattern at these four restriction endonucleases or a C pattern for the same four restriction endonucleases (all sauger were scored with the B pattern). The C pattern was only detected within the New

Cumberland (RM 54) and Pike Island (RM 84) tailwaters while the A pattern was detected in all tailwaters and Lake Erie (Table 3). Two different banding patterns were scored at *RSA I* for walleye with the A mitochondrial type and two other distinct patterns were scored for walleye with the C mitochondrial type resulting in a total of four composite mitochondrial patterns for walleye (Table 2). A fifth composite mitochondrial pattern was scored for sauger (Table 2). Although size variation in the Dloop/12s RNA mitochondrial regions was observed among individual walleye the observed differences between the A and C banding patterns were not a result of that variation.

Protein Electrophoretic Analysis

A total of 15 loci were used to assess the genetic variation in walleye from eight locations on the Ohio River, one location from Lake Erie, and one location from Lake Winnebago, WI (Table 3). Six of 15 loci were polymorphic (*AAT-B*, *ADH-1*, *CBP-1*, *FBP-1*, *sIDHP-B*, and *MDH-B*) within individuals identified as pure walleye. Four of the loci (*ADH-1*, *CBP-1*, *sIDHP-B*, and *MDH-B*) were polymorphic in all populations except for *ADH-1* in Racine, OH. Genetic distance among populations was calculated with Rogers (1972) coefficient using the program BIOSYS-1 (Swofford and Selander, 1981). The resulting distance matrix was used to determine phenetic relationships among populations using a UPGMA cluster analysis (Sneath and Sokal, 1973).

Genotype frequencies for those walleye that were scored with the A mitochondrial banding pattern were calculated separately from those scored with the C pattern and analyzed as independent groups (54A, 54C, 84A, and 84C). Allele frequencies calculated between the A and C types of walleye from river mile 54 varied the most at two loci (*CBP-1* and *IDHP-B*), while allele frequencies between the A and C

types of walleye from river mile 84 varied the most at *IDHP-B* (Table 4). The differences between the allele frequencies at *CBP-1* and *IDHP-B* for the A and C types of walleye resulted in the 54C and 84C walleye clustering distantly to the 54A and 84A walleye in comparison to the other walleye analyzed (Figure 1). Four major groups were identified by the clustering analysis (Figure 1): first, walleye from river mile 54 and 84 with the A mitochondrial type plus walleye from river mile 162 and 342 grouped together; secondly, walleye from river mile 436 and 606 grouped with walleye from Lake Erie; thirdly, walleye from L. Winnebago, WI population grouped alone; and lastly, the walleye from RM 54 and 84 with the C mitochondrial type grouped together (Figure 1). Walleye from Racine, OH (RM 238) and McAlpine, IN (RM 720) were not included in the analysis because of the low sample sizes analyzed from those sites.

Sequence Analysis of the cytochrome-B mitochondrial genes

Samples of walleye from RM 54 and 84 with the A and C mitochondrial types, walleye from L. Erie (all have A mitochondrial type), and walleye from the Illinois River (all have A mitochondrial type) were sequenced to determine the percent sequence divergence among walleye with the A and C mitochondrial types and among walleye from the different sampling locations. Results from this analysis calculated mean sequence divergence between walleye with the A and C types at 3.5% while sequence divergence among walleye with the A type was less than 0.5%.

Discussion

Allozyme analysis using three loci was conducted in a previous study to assess how accurate field identification of walleye was using morphological characteristics. That analysis identified that some individuals that were tentatively identified as walleye were in fact F1 or Fx hybrid saugeye or pure sauger. All samples identified as F1/Fx hybrids were then analyzed using an mtDNA analysis, revealing that the F1 saugeye were being produced from a walleye female and sauger male. The allozyme analysis identified that Fx saugeye individuals were produced from an F1 or Fx saugeye backcrossing with a walleye. The mtDNA analysis could not distinguish between a saugeye male that had backcrossed with a pure walleye female or a saugeye female that backcrossed with a walleye male because both Fx individuals would have walleye mtDNA. The walleye and sauger samples identified by the allozyme analysis from eight tailwaters of the Ohio River have also been analyzed using an mtDNA analysis in this study.

Two different types of mitochondrial DNA (A and C patterns) were found in walleye from two upper reaches (river mile 54 and 84) of the Ohio River where samples were collected. All other walleye samples collected had only the A mitochondrial type. Several hypotheses can explain the presence of the C mitochondrial type that was detected in the Ohio River. First, walleye with the C mitochondrial type may be remnants of a native population of walleye from the Ohio River or secondly, walleye with the C type may have been introduced into the Ohio River from an outside source. Stocking records from the Ohio Department of Wildlife indicate that walleye have only been stocked into the Ohio River from Lake Erie. This analysis has found only the A

mitochondrial type in Lake Erie, indicating the walleye with the C mitochondrial type in the Ohio River are not a result of stocking Lake Erie walleye. However, an unknown stocking of walleye with the C mitochondrial type from an outside source is still possible and, therefore, the C mitochondrial type might not be native to the Ohio River. The presence of walleye with the A mitochondrial type at river mile 54 and 84 could be a result of upstream or downstream migration of walleye with the A mitochondrial type that were originally stocked from Lake Erie or from some other unknown source.

The A mitochondrial type may have been native to parts of the Ohio River, perhaps only in the lower regions. Walleye with the A mitochondrial type may have expanded upriver through competition with walleye with the C. Secondly, walleye with the C mitochondrial type may never have been present in the lower reaches of the Ohio River. The low sample sizes of walleye from each tailwater in conjunction with the number of walleye that have been stocked, limits our ability to assess if there were or were not any walleye with the C mitochondrial pattern in the lower reaches. The lowest reaches of the Ohio River were probably influenced by migration of walleye from the Mississippi River that did not have the C mitochondrial pattern; therefore an intergrade zone potentially existed between an Ohio River form of walleye and a Mississippi River form. The lack of the C mitochondrial type in the lower reaches may then be reflective of a historic distribution of walleye based on migration in and out of the Ohio River.

If walleye with the A and C mitochondrial types randomly mate then there would be no difference in allele frequencies expected among individuals with the two types. Allozyme results indicate that this in not the case; individuals are have been mating assortatively – at least to some extent. What we do not know is the allele frequency of

A and C type walleye prior to introgression. As a result, we cannot estimate the level of introgression (if any) that has occurred.

Management Recommendations

1. Assuming the C mitochondrial form is not present as the result of unknown stocking from an outside source, upper reaches of the Ohio River where the C mitochondrial type has been found could be stocked with walleye that have the C mitochondrial form. Walleye that are collected should be screened and only those with the C mitochondrial type could be used as brood stock for those areas. Prior to that decision, further inspection of walleye from other watersheds or drainage basins should be conducted to determine if the C mitochondrial type is widespread in the upper basin, providing evidence that the C mitochondrial type is more likely native to the Ohio River than from an outside source.

2. Middle and lower reaches of the Ohio River could be stocked with walleye with the A mitochondrial type only. The lack of walleye detected with the C mitochondrial type and the inability to positively determine the native stock of walleye to this region would suggest the type that is currently found there should be used. Walleye with the C mitochondrial type may not have ever been present historically because they were not physically suited for the conditions in these stretches of the Ohio River.

3. Assuming the C mitochondrial type is native to the Ohio River and conditions of the water bodies being stocked are similar to the areas in the upper reaches, upstream

regions where few or no walleye are present could be stocked with walleye with C type mitochondrial DNA. Performance of these fish, however, should be monitored overtime to determine their sustainability in the newly introduced areas.

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Table 1. Sample sites and the number of fish visually identified as walleye and collected. The number of walleye, F1, Fx, and sauger analyzed using an allozyme analysis and mtDNA analysis. All samples were not analyzed due to poor amplification of those individuals.

Tailwater	River Mile	Total # WAE Collected*	# WAE Analyzed Allozymes	# WAE Analyzed mtDNA	# F1's, Fx's, SAU Analyzed mtDNA
Lake Erie	---	5	0	5	0
New Cumberland, OH	54	43	30**	30	2
Pike Island, WV	84	30	21**	21	3
Willow Island, OH	162	39	39	15	16
Racine, OH	238	4	4	3	1
Greenup, OH	342	40	40	14	8
Meldahl, OH	436	9	9	6	5
Markland, KY	606	16	16	14	4
McAlpine, IN	720	6	6	4	1

* Total number of walleye is based on the results of the allozyme analysis identifying walleye from F1 and FX hybrids.
** Only walleye were analyzed from New Cumberland and Pike Island when mtDNA data was available.

Table 2. Composite haplotypes detected in walleye, F1/ Fx hybrids, and sauger in eight tailwaters of the Ohio River and Lake Erie.

	Restriction Endonucleases						
	BSTU I	*DDE I*	*DPN II*	*HAE III*	*HHA I*	*MSP I*	*RSA I*
1	A	A	A	A	A	A	A
2	A	A	A	A	A	A	C
3	A	C	C	C	C	A	D
4	A	C	C	C	C	A	E
5	B	B	B	B	B	B	B

Table 3. Number of individual walleye, F1/Fx hybrids, and sauger scored with the same composite haplotypes 1-5 from eight tailwaters of the Ohio River and L. Erie.

		Walleye					F1/ Fx hybrids and Sauger				
Tailwater	River Mile	1	2	3	4	5	1	2	3	4	5
Lake Erie	----	5	0	0	0	0	0	0	0	0	0
New Cumberland, OH	54	13	6	6	5	0	1	0	1	0	0
Pike Island, WV	84	11	1	2	7	0	2	0	0	1	0
Willow Island, OH	162	11	3	0	0	0	3	5	0	0	8
Racine, OH	238	3	0	0	0	0	1	0	0	0	0
Greenup, OH	342	13	1	0	0	0	5	3	0	0	0
Meldahl, OH	436	4	2	0	0	0	4	1	0	0	0
Markland, KY	606	13	1	0	0	0	4	0	0	0	0
McAlpine, IN	720	3	1	0	0	0	1	0	0	0	0

Table 4. Allele frequencies scored for 11 allozyme loci in walleye from eight tailwaters of the Ohio River, L. Erie, and L. Winnebago.

	Populations listed by River Mile											
	54A N=19	54C N=11	84A N=12	84C N=8	162 N=39	238 N=4	342 N=40	436 N=9	606 N=16	720 N=6	Erie N=30	Winnebago N=30
AAT-M												
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
AAT-B												
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.944	1.000	1.000	1.000	1.000
115	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.056	0.000	0.000	0.000	0.000
ADH-1												
-60	0.026	0.000	0.125	0.000	0.077	0.000	0.083	0.056	0.062	0.167	0.259	0.167
-100	0.974	1.000	0.875	1.000	0.923	1.000	0.917	0.944	0.938	0.833	0.741	0.833
AK-1												
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
CBP-1												
100	0.526	0.227	0.500	0.188	0.551	0.625	0.575	0.500	0.719	0.833	0.552	0.833
160	0.474	0.773	0.500	0.813	0.449	0.375	0.425	0.500	0.281	0.167	0.448	0.167
FBP-1												
85	0.000	0.000	0.000	0.000	0.000	0.125	0.000	0.000	0.062	0.000	0.000	0.000
100	1.000	1.000	1.000	1.000	1.000	0.875	1.000	1.000	0.938	1.000	1.000	1.000
IDHP-A												
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
IDHP-B												
75	0.500	0.773	0.667	0.688	0.590	0.500	0.444	0.278	0.375	0.333	0.310	0.483
100	0.500	0.227	0.333	0.313	0.410	0.500	0.556	0.722	0.625	0.667	0.690	0.517
LDH-1												
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
MDH-M												
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
MDH-A												
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Table 4 continued.

	Populations listed by River Mile											
	54A N=19	54C N=11	84A N=12	84C N=8	162 N=39	238 N=4	342 N=40	436 N=9	606 N=16	720 N=6	Erie N=30	Winnebago N=30
MDH-B												
100	0.500	0.364	0.667	0.250	0.577	0.125	0.662	0.778	0.750	0.417	0.741	0.883
120	0.500	0.636	0.333	0.750	0.423	0.875	0.338	0.222	0.250	0.583	0.259	0.117
PGDH-1												
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
PGM-A												
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
SOD-1												
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Figure 1. UPGMA cluster analysis of allozyme data.