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GENETIC STRUCTURE AMONG FOUR POPULATIONS OF PADDLEFISH, *POLYODON SPATHULA* (ACTINOPTERYGII: ACIPENSERIFORMES: POLYODONTIDAE), BASED ON DISOMIC MICROSATELLITE MARKERS

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Background. The paddlefish, *Polyodon spathula* (Walbaum, 1792), is an important species for commercial and recreational fisheries throughout the central United States. Populations have declined in many areas due to river modification, loss of spawning habitat, pollution, and over-exploitation. Assessing genetic diversity of a species is an important consideration for developing conservation plans. The goal of this research was to perform a broad range survey of paddlefish diversity by evaluating populations from geographically distant major rivers of the United States of America.

Materials and methods. Paddlefish samples were collected from four sites including the Alabama River, Red River, Yellowstone/Missouri River, and Ohio River. Eight microsatellite loci (*PspD102*, *PspD111*, *PspB105*, *PspD9*, *PspD8*, *PspC6*, *PspH26*, and *PspC10*) that displayed disomic inheritance patterns were used for the amplification of alleles.

Results. Average allelic richness of four sites ranged from 7.50 ± 1.36 to 5.46 ± 0.91. Average expected heterozygosity ranged from 0.717 ± 0.085 to 0.591 ± 0.093, the average observed heterozygosity assumed the values from 0.711 ± 0.115 to 0.585 ± 0.087. A moderate level of between population diversity was observed with an overall F_{st} value of 0.0702. Hardy–Weinberg equilibrium revealed that seven loci in the four populations were in equilibrium. The four populations were clustered to two categories by cluster analysis (UPGMA) based on F_{st} and $\delta\mu^2$ distance.

Conclusion. Four studied paddlefish populations exhibited relatively low levels of genetic diversity and close relative relations, but still had some differentiation among the populations. The genetic distance and F_{st} revealed that the Ohio River, Red River and Yellowstone/Missouri River populations belong to the same branch, while the Alabama River population from another branch.

Keywords: *Polyodon*, molecular marker, geographic structure, genetic variety

INTRODUCTION

The paddlefish, *Polyodon spathula* (Walbaum, 1792), is an ancient, planktivorous freshwater species that inhabits large rivers and lakes throughout much of the Mississippi River drainage and smaller rivers of the Gulf slope drainages in North America (Carlson and Bonislawsky 1981, Jennings and Zigler 2000). Paddlefish migrate upstream and based on environmental parameters (e.g., temperature, substrate, and flow) select areas for successful spawning and survival of early life stages (Lein and DeVries 1998, Stancill et al. 2002). The periphery of the Mississippi River including the Yellowstone River, Missouri River, Red River, and Alabama River provide the essential ecological attributes necessary for paddlefish reproduction and recruitment (Scarnecchia et al. 1996, Braaten et al. 2009).

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Paddlefish have long supported commercial and recreational fisheries throughout the central United States (Pasch and Alexander 1986, Graham 1997). With the decline of the Caspian Sea sturgeon fishery, the traditional source of caviar, additional pressure has been placed on wild paddlefish stocks for their roe (Carlson and Bonislawsky 1981). In many areas, wild paddlefish populations have declined due to river modification, loss of spawning habitat, pollution and over-exploitation (Jennings and Zigler 2000). In 1992, paddlefish was placed on the United Nation's Convention on International Trade in Endangered Species (CITES) act in an effort to curtail illegal trade of paddlefish and their parts (Allardyce 1991*) and support conservation plans (Epifanio et al. 1996).

Genetic diversity of a species is an important consideration for conservation and management practices. Molecular techniques can be utilized to assess patterns of genetic diversity and identify populations that require greater conservation efforts (Johnson et al. 2001). In paddlefish, many molecular markers systems have been used to study population genetics. Previous studies focusing on protein polymorphism and mitochondrial DNA markers (mtDNA) demonstrated relatively low levels of genetic variability within respective analyzed populations of paddlefish (Epifanio et al. 1996, Szalanski et al. 2000).

High polymorphism typically obtained with microsatellite markers has provided more acute information for detecting subtle differences among geographically proximal populations. Heist and Mustapha (2008) surveyed genetic variation among 12 geographic locations of paddlefish collected throughout the range of the species at five microsatellite loci; three with disomic inheritance and two with tetrasomic inheritance. Heist and Mustapha (2008) demonstrated that nearly all analyzed populations exhibited significant genetic heterogeneity with the most distinct population being the Tombigbee River followed by Grand Lake and Bayou Nezpique. Although the levels of genetic diversity of paddlefish have been well studied, further investigation using microsatellites is needed. In this research, we used 8 microsatellite loci to analyse the genetic diversity and differences of four populations, we hope this study will help to further understand the paddlefish population structure, differences and to evaluate the status of germplasm resource, and provide a theoretical support for conservation and utilization of paddlefish, *Polyodon spathula*.

MATERIALS AND METHODS

Sample collection and storage. Samples of paddlefish, *Polyodon spathula*, were collected from four sites including the Alabama River (Wilcox county, Elmore, Montgomery, and Talladega counties, Alabama) (AL; $n = 30$), Red River (Oklahoma, river mile 718, Louisiana, river miles 152, 162, and 168) (RR; $n = 24$), Yellowstone/Missouri River (McKenzie county, North Dakota) (YM; $n = 29$), and Ohio River (Jefferson county, Kentucky) (OH; $n = 21$) (Fig. 1). Collection of the fish was performed in accordance with permits issued by Fish and Wildlife Services of Kentucky, Alabama, Oklahoma, and North Dakota State. Fin clips were collected from individuals and immediately placed in 95% ethanol for transport to Kentucky State University. All samples were collected by State Fish and Wildlife personnel working in the areas surrounding sample sites with the exception of the Ohio River samples; which were collected by Kentucky State University personnel.

DNA extraction, microsatellite primer design and amplification. Total genomic DNA of *Polyodon spathula* was extracted using the Promega Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, WI); mouse tail extraction protocol. Total DNA concentration was determined using a GeneQuant™ *pro* RNA/DNA Calculator Spectrophotometer (GE Healthcare-Life Sciences, Piscataway, New Jersey). A portion of the total DNA was diluting to10 ng · μL^{-1} for use as a template source in PCR reactions.

A microsatellite library was developed by Genetic Identification Services (Chatsworth, CA) using the method described by Jones et al. (2002). Polymerase chain reactions (PCR) were performed using Techne® TC-215 gradient thermal cycler (Bibby Scientific, UK). Each reaction contained 1.5 mM $MgCl_2$ (Promega), 5X PCR buffer (Promega), 200 µM of each dNTPs (Promega), 0.25 units of *Taq* DNA polymerase

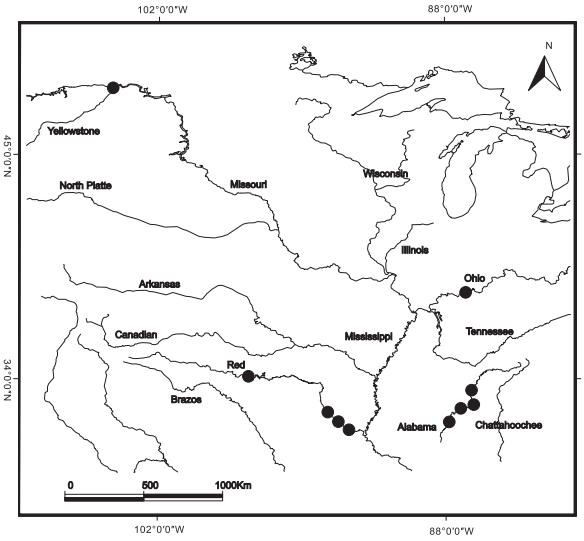

Fig. 1. Map of the Alabama River, Red River, Yellowstone/Missouri River, and Ohio River; Circles indicate the sampling sites of paddlefish, *Polyodon spathula* (Alabama River: Wilcox County, Elmore, Montgomery, and Tallapoosa counties, Alabama; Red River: Oklahoma, river mile 718, Louisiana, river miles 152, 162, and 168; Yellowstone/Missouri River: McKenzie County, North Dakota; Ohio River: Jefferson County, Kentucky)

* Allardyce D.A. 1991. Endangered and threatened wildlife and plants: Notice of finding on petition to list the paddlefish. Department of Interior, US Fish and Wildlife Service, Final Report 50 CFR, Part 17.

(Promega), 5.0 pM of each forward and reverse primers (Integrated DNA Technologies, Coralville, IA), 20 ng of template DNA and PCR H_2O to a final volume of 10 µL. PCR cycling conditions consisted of an initial denaturing step of 94ºC for 2 min followed by 35 cycles of 30 s at 94ºC, 1 min at annealing temperature (Table 1), and 45 s at 72ºC with a final extension step of 7 min at 68ºC.

Following amplification, 0.5 µL of PCR product was mixed with 0.25 µL of GenScan™ 500 LIZ™ internal size standard (Applied Biosystems) and 9.25 µL HiDi ™ Formamide (Applied biosystems) and denatured at 95ºC for 6 min then chilled on ice for 2 min. Amplified products were resolved via capillary electrophoresis on an ABI 3130 Genetic Analyzer (Applied Biosystems). Fragment sizes were determined using GeneMapper® software version 3.5 (Applied Biosystems).

Statistical analyses. Used Micro-checker 2.2.3 (Van Oosterhout[*]) to test for null alleles. Observed heterozygosity (H_o) and unbiased expected heterozygosity (H_e) (Nei 1978) were computed using TFPGA (ver. 1.3[**]). The number of observed alleles (A) and allelic richness (A_r) were computed using FSTAT (ver. 2.9.3[***]). Values of A_r and H_e were tested for significance ($P \leq 0.05$) between populations using Wilcoxon signed rank test. The number of private alleles (A_p) and private allelic richness (A_{rp}) were computed using HP-Rare (Kalinowski 2005). Values of A_r and A_{rp} were computed based on a rarefaction size of $2n$, where $n = 21$, the smallest single locus sample size examined (Ohio River).

Deviation from Hardy–Weinberg equilibrium (F_{is}) was quantified by Weir and Cockerham's (1984) using FSTAT. Probability values (HW) (P-value) were estimated by exact tests in GENEPOP version 4.0 (Rousset 2008) using a Markov Chain Randomization method (Guo and Thompson 1992) with the following parameters; dememorization = 40 000, batches = 50, and iterations per batch = 40 000.

Population differentiation (F_{st}) overall populations and between population pairs of *Polyodon spathula* were quantified using Weir and Cockerham's (1984) θ in SPAGeDi version 1.2 (Hardy and Vekemans 2002). Significance ($P \leq 0.05$) of pair-wise F_{st} values differing from zero was estimated by permutation tests (1000 random permutations of individuals and genes). Homogeneity tests of genetic differentiation between all population pairs were tested using a Markov Chain Randomization method in GENEPOP employing the same parameters described above. Significance levels ($P \leq 0.05$) were adjusted by sequential Bonferroni correction to account for multiple hypotheses testing (Rice 1989). The estimated number of migrants (Nm) between populations was calculated in GENEPOP based on the correction for sample size (Barton and Slatkin 1986).

The microsatellite specific analogue of Nei's (1978) standard genetic distance (Ds) $\delta\mu^2$ (Goldstein and Pollok 1997) was calculated between all population pairs using SPAGeDi. Unweighted Pair Group Method with Arithmetic Mean (UPGMA) dendrograms were con-

Table 1

Characterization of eight disomic microsatellite loci for the paddlefish, *Polyodon spathula*

Locus	Primer sequence (5′–3′)	Fluorophore	No. of Alleles	Allele size range [bp]	T_a [ºC]
Psp D102	F CAGCAACACTAAAGGAAACTTG R TGGGAACTCACTATTATCAAAC	FAM	14	280–352	48
Psp D111	F GCTTGTGCCATTCTGTCTAC R TTGCTGTCTTTATCAAACCAG	HEX	18	192–260	47
Psp B105	F GCAAAGTCACAAAATGGTCAG R GTTTCCTGTAGTCACCCTCCAACTC	HEX	12	189–225	49
Psp D9	F CATTATCGCTGCTCTCAATATC R AGCTTAGTTCTGGGTTTAGACC	FAM	10	121–161	47
Psp D8	F ATGGGCTCTACACAGTGATTC R AAACCCTCACCAAGTTAAATTC	FAM	13	181–277	48
Psp C6	F CCGAGTGTTGTGTTTCTTTTG R AAGTTGCTGCCTTTTGTCTTC	FAM	3	182–194	50
Psp H26	F TCGGTGTTTGTGTGTGTGTATGC R GTTTGGTTCCAGTTTCGCTCATCC	FAM	9	130–162	53
Psp C10	F AAGGGGCTAATGAGCAATG R AAGTGGGGGTGCTGAAAG	FAM	2	219–223	49

T_a = annealing temperature.

[*] Van Oosterhout C., Hutchinson WF, Wills DPM, Shipley P 2005. Micro-checker 2.2.3 University of Hull, HU67RX, UK.
[**] Miller M. P. 1997. TFPGA version 1.3 Northern Arizona Univ., Flagstaff, AZ, USA.
[***] Goudet D. B. 2002. FSTAT version 2.9.3 UNIL., Lausane, Vaud, Switzerland.

structed based on F_{st} and $\delta\mu^2$ distance using PHYLIP (ver. 3.68*). Dendrograms were visualized and edited in Tree Explorer (ver. 2.12**).

RESULTS

Twenty microsatellite loci were initially screed and eight loci were selected for further use in this study based on quality of amplification and disomic inheritance (Table 1). A total of 81 alleles were observed with an average of 10.1 alleles per locus, ranging from 2 alleles at Psp C10 and to 18 alleles at Psp D111. Expected heterozygosity was significantly ($P \leq 0.05$) lower in the Red River and Alabama River populations of *Polyodon spathula* when compared to the Yellowstone/Missouri River population. Expected heterozygosity of the Ohio River population was intermediate and not significantly ($P \leq 0.05$) different from the Yellowstone/Missouri River and Red River populations but significantly higher than the Alabama River population (Table 2), genetic diversity of the Alabama River population is lower than other population.

Significant ($P \leq 0.05$) deviation from Hardy–Weinberg equilibrium was observed in four of thirty two single locus exact tests before sequential Bonferroni correction; three deviations occurred at locus *PspD111* in the Ohio River, Red River, and Yellowstone/Missouri River populations and one deviation occurred at locus *PspC10* in the Yellowstone/Missouri River population. Following sequential Bonferroni correction, only the locus *PspD111* deviations in the Ohio River and Red River populations were significant, which were caused by null alleles, the microchecker analysis showed locus *PspD111* had null alleles. Overall locus exact tests revealed significant departure from Hardy–Weinberg equilibrium in Ohio River and Yellowstone/Missouri River populations before sequential Bonferroni correction and in the Ohio River population following sequential Bonferroni correction.

Differentiation among populations of *Polyodon spathula* was moderate with an overall F_{st} value of 0.0702; which was significantly different from zero ($P < 0.0001$). All pair-wise comparisons of F_{st} differed significantly from zero before and after sequential Bonferroni correction with the exception of the Red River and Yellowstone/Missouri River population comparison ($F_{st} = 0.009$ $P = 0.0889$) (Table 3). The highest level of

Table 2

Genetic diversity indices of four geographic populations of paddlefish, *Polyodon spathula* from Noth America

Parameter	Population (*n*)			
	Ohio River (21)	Alabama River (30)	Red River (24)	Yellowstone/Missouri River (29)
A	7.00 ± 1.27	5.88 ± 1.03	7.75 ± 1.45	7.63 ± 1.16
A_r	7.00±1.27[a]	5.46 ± 0.91[b]	7.50 ± 1.36 [a]	7.250 ± 1.09[a]
A_p	0.50 ± 0.27	0.63 ± 0.38	0.38 ± 0.18	0.75 ± 0.25
A_{rp}	0.54 ± 0.27	0.62 ± 0.36	0.42 ± 0.15	0.67 ± 0.20
H_o	0.673 ± 0.066	0.585 ± 0.087	0.648 ± 0.082	0.711 ± 0.115
H_e	0.691 ± 0.069[ab]	0.591 ± 0.093[c]	0.676 ± 0.091[bc]	0.7173 ± 0.085 [a]
F_{is}	0.028	0.013	0.044	0.099
HW	<0.0001[$$]	0.778	0.095	0.017[$]

n = sample size; *A* = number of observed alleles; A_r = allelic richness; A_p = number of private alleles; A_{rp} = private allelic richness; H_o = observed heterozygosity; H_e = expected heterozygosity; F_{IS} = Weir and Cockerham's (1984) estimators of inbreeding coefficient; HW = *P*-value for test of Hardy–Weinberg equilibrium; [$]Value is significant at the α = 0.05 level before sequential Bonferroni correction; [$$]Value is extremely significant following sequential onferroni correction; Different superscript lowercase letters show significant differences ($P < 0.05$).

Table 3

Pair-wise estimates of F_{st} (above diagonal) and distance (Goldtein 1997) (below diagonal) among four geographically separate populations of paddlefish, *Polyodon spathula*, sampled from North American rivers

Location (river)	Ohio	Red	Yellowstone/Missouri	Alabama
Ohio	—	0.0161[$]	0.0170[$]	0.1381[$]
Red	0.0000	—	0.0094	0.1194[$]
Yellowstone/Missouri	0.4624	2.0980	—	0.0952[$]
Alabama	24.0443	27.7314	27.6027	—

[$]Value is significant from zero at the α = 0.05 level following sequential Bonferroni correction.

* Felsenstein J. 2008. PHYLIP version 3.68 UW., Seattle, WA, USA.
** Tamura K. 1999. TreeExplorer. version 2.12. PSU, University Park, PA, USA.

differentiation based on F_{st} was found between the Ohio River and Alabama River populations (F_{st} = 0.138), however, the greatest distance based on $\delta\mu^2$ was found between the Red River and Alabama River populations ($\delta\mu^2$ = 27.731).

Of the 48 pair-wise comparisons of genetic differentiation, 27 were significant ($P \leq 0.05$) before Bonferroni correction and 19 were significant following Bonferroni correction. Significant heterogeneity was observed between the Alabama River population and all other populations at loci *PspD102, PspD111, PspD9, PspD8, PspB105*, *and PspH26*. In addition, the Yellowstone/Missouri River population was significantly different from the Ohio River population at locus *PspD9*.

Dendrograms constructed from F_{st} and $\delta\mu^2$ both demonstrated the dissimilarity of the Alabama River population of *Polyodon spathula* in comparison with the other sampled populations (Fig. 2). Among the Ohio River, Yellowstone/Missouri River and Red River populations the dendrogram based on F_{st} (Fig. 2A) more closely associated the Red River population with the Yellowstone/Missouri River population while the dendrogram based on $\delta\mu^2$ (Fig. 2B) more closely associated the Red River population with the Ohio River population.

DISCUSSION

The results of the current experiment clearly demonstrate the uniqueness of paddlefish, *Polyodon spathula*, from the Alabama River drainage. Similar results have been previously demonstrated by protein polymorphism (Carlson et al. 1982), mtDNA (Epifanio et al. 1996) and microsatellites (Heist and Mustapha 2008). It is documented that the Alabama River drainage has been separated from the Mississippi River drainage for 25 000–30 000 years (Ramsey 1965*) and it would be expected that this duration of isolation would result in significant genetic divergence.

Perhaps the most demonstrative index of the differentiation or divergence in the Alabama River population is the frequency of private alleles. Private alleles were found in all populations; with a similar number of private allele found in each population. However, the observed gene frequency of private alleles was much higher in the Alabama River population than any other population. In the Alabama River population, the three private alleles found at locus *PspD111* has a sum population frequency of 0.38 and the one private allele at *PspD8* had an observed population frequency of 0.20. The highest private allele frequency in any other sampled population was

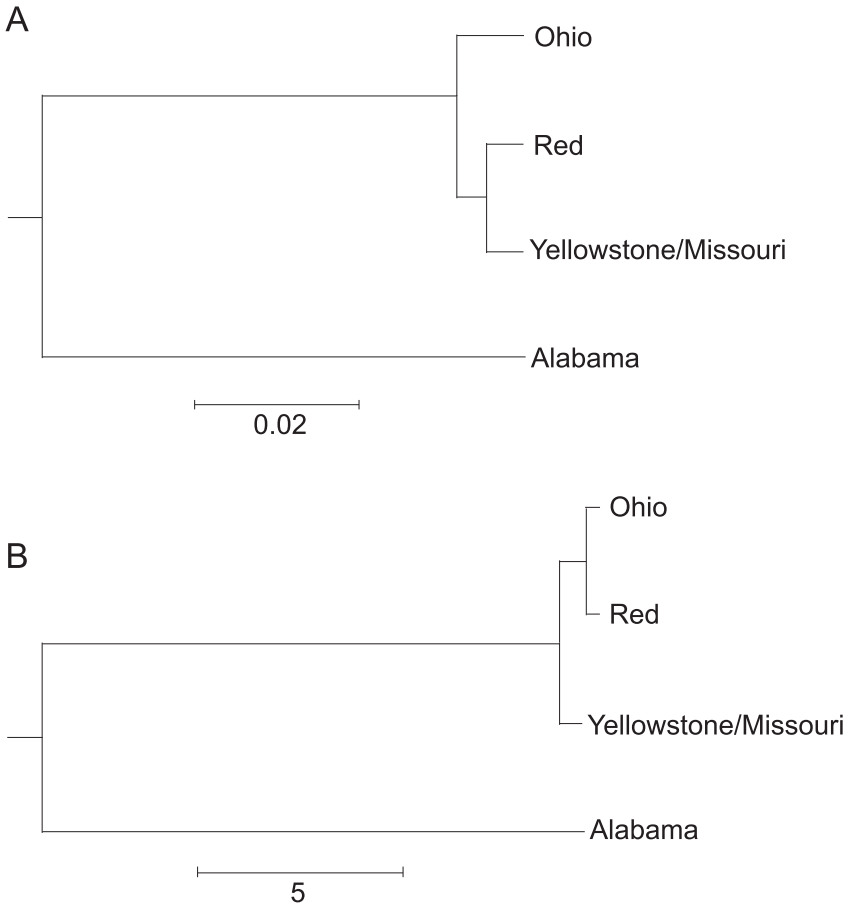

Fig. 2. UPGMA dendrograms based on F_{st} (**A**) and distance (**B**) for four geographic populations of paddlefish, *Polyodon spathula*, at eight disomic microsatellite loci

* Ramsey J.S. 1965. Zoogeographic studies on the freshwater fish fauna of rivers draining the southern Appalachian region. Doctoral dissertation. Tulane University, New Orleans, Louisiana.

0.05 at locus *PspD9* in the Yellowstone/Missouri River population. Additionally, although low frequency, the private allele in the Yellowstone/Missouri River population at locus *PspC6* is noteworthy as only three alleles were found across all the sampled populations at this locus.

Heist and Mustapha (2008) evaluated microsatellite diversity among 12 geographic locations of paddlefish, *Polyodon spathula*, and found significant heterogeneity among most sampled populations. The authors suggested that significant difference in allelic frequency developed recently due to the construction of dams which limit gene flow. In the present study, the approach to assessing diversity was to evaluate paddlefish populations that were geographically far distant. In contrast to the findings of Heist and Mustapha (2008), relatively low levels of diversity were found between the Ohio River, Red River and Yellowstone/Missouri River populations. Based on F_{st}, the Red River and Yellowstone/Missouri River populations were not significantly different and essentially fixed for the same alleles. Based on, there was no relative distance between the Ohio River and Red River populations. The Ohio River, Red River and Yellowstone/Missouri River had significantly higher levels of within population diversity compared to the Alabama River population based on allelic richness. This difference is likely caused by reproductive isolation due to dam construction.

Due to the increased pressure on wild stocks of paddlefish, *Polyodon spathula*, genetic monitoring will be vital for successful conservation and management. Attempts should be made to preserve low frequency and unique alleles that may be lost due to reproductive isolation and inbreeding. Also, attempts should be made to mitigate outbreeding and preserve the uniqueness of Alabama River drainage populations. An important aspect of genetic management will be to monitor hatchery propagation of paddlefish. Special consideration should be given to the effective population size and the geographic source of brood fish. Finally, aquaculture of paddlefish should be a focus to decease pressure on wild stocks for caviar production.

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