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NOTES

Population Genetic Structure of Nebraska Paddlefish Based on Mitochondrial DNA Variation

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Abstract.—Eighty-three paddlefish *Polyodon spathula* that were collected from 1995 to 1999 from the Missouri River Galvins Point Dam tailwater were analyzed for genetic variation in the mitochondrial DNA d-loop region. Additional samples from Montana, South Dakota, and Louisiana were used for comparative purposes. To facilitate the efficient analysis of numerous paddlefish samples, we applied a method that employs polyacrylamide gel electrophoresis (PAGE) to resolve restriction fragment length polymorphisms (RFLPs) amplified by polymerase chain reaction (PCR). DNA sequencing of 10 paddlefish revealed 22 polymorphic sites. Polymerase chain reaction–RFLP analysis of 93 paddlefish using three restriction enzymes detected six of the polymorphic sites and revealed six distinct haplotypes. All of the observed haplotypes were found in the Missouri River Galvins Point Dam tailwater. No temporal differentiation was observed among the 1995, 1998, and 1999 samples from the Missouri River Galvins Point Dam tailwater. Polymerase chain reaction–RFLP, resolved with PAGE, provided an efficient method for population genetic analysis of paddlefish.

The paddlefish *Polyodon spathula* Walbaum is an ancient fish that is native to the Missouri River, the Mississippi River, and several Gulf Coast drainages of North America (Gengerke 1986; Russell 1986). Paddlefish populations have declined along with a reduction in their range because of impoundment channel alterations and overharvesting (Gengerke 1986; Russell 1986). On the Missouri River, dams have interrupted migration routes and flooded or fragmented critical habitats (Unkenholz 1986). The decline of paddlefish populations has caused them to be listed as a species of concern in several states (Johnson 1987); they were also listed in the Convention on International Trade in Endangered Species in 1992 (Allardyce 1992). A report of the National Paddlefish and Sturgeon Steering Committee (1993) identified the loss of genetic variation as one of the committee's primary concerns. However, information about paddlefish population genetic structure is incomplete. Previous allozyme studies (Carlson et al. 1992; Fries and Hutson 1993; Epifanio et al. 1996) and mitochondrial DNA (mtDNA) restriction fragment length polymorphism (RFLP) analysis (Epifanio et al. 1996) revealed low levels of allozyme allelic and mtDNA haplotype variation within and among paddlefish populations sampled from up to nine tributaries (Epifanio et al. 1996). The biochemical and molecular methods previously employed are not optimal for genetic analysis of numerous alcohol-preserved, field-collected samples.

The objective of this study was to characterize mtDNA d-loop genetic variation from temporally sampled paddlefish collected from the Missouri River Galvins Pt. dam tailwater. Additional populations isolated by geographical range or by dams were included for comparative purposes. To facilitate the efficient analysis of numerous paddlefish samples, we have applied a method that employs polyacrylamide gel electrophoresis (PAGE) to resolve RFLPs amplified by polymerase chain reaction (PCR).

Methods

Fin tissue samples (approximately 10 × 30 mm) from individual paddlefish caught and released from the Galvins Pt. Dam tailwater on the Missouri River were obtained during 1995, 1998, and 1999; samples were preserved by freezing at −70°C or by immersion in 95% ethanol (Table 1). Muscle and egg samples that had been preserved by freezing were also obtained from paddlefish collected in 1995 from South Dakota, Louisiana, and Montana (Table 1). DNA was extracted from a 5 × 10–mm section of tissue or from individual eggs using a phenol/chloroform extraction procedure (Taylor et al. 1996). The mtDNA d-loop region was amplified using the primers Proline-tRNA-2 (5′-ACCCTTAACTCCCAAAGC-3′) and Phenyl-tRNA-1 (5′-GTGTTATGCTTTAGTTAAGC-3′) (Bernatchez et al. 1992) to generate an approximately 875-base

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TABLE 1.—Three-enzyme haplotypes of paddlefish, in the order *Nla* III, *Rsa* I, and *Tsp509* I. Abbreviations are as follows: LA = Mermentau River, Louisiana; SD = Missouri River, Lyman County, South Dakota; MT = Yellowstone River, Dawson County, Montana; NE = Missouri River, Galvins Point Dam tailwater, Cedar County, Nebraska; numerals signify 1995, 1998, 1999, respectively.

Haplotype	Sample						
	LA	SD	MT	NE95	NE98	NE99	*N*
AAA	0	0	1	5	8	6	20
AAD	0	0	0	2	1	3	6
ABA	0	1	1	3	6	8	19
BAC	0	0	1	1	1	8	11
CAA	0	0	0	1	0	0	1
CAB	2	0	4	7	5	18	36
N	2	1	7	19	21	43	93

pair (bp) amplicon. Amplification conditions were as follows: 35 cycles of 94°C for 45 s, 56°C for 45 s, and 72°C for 90 s. For DNA sequencing, PCR products were purified using Micron 50 microconcentrator (Amicon, Inc., Beverly, Massachusetts) and resuspended to a volume of 10.0 μL using nanopure water. Purified and concentrated DNA, approximately 2.5 ng/100 bp, was sent to the DNA Sequencing Facility at Iowa State University (Ames, Iowa) for direct sequencing in both directions. Consensus sequences for each individual were derived using the Genetics Computer Group (GCG; Madison, Wisconsin) GAP program. The GenBank accession numbers for each sequence are AF176331 to AF176340. Sequences were aligned with GCG PILEUP using shovelnose sturgeon *Scaphirhynchus platorynchus* (Rafinesque) (GenBank accession number AF176341) as the outgroup taxon. Parsimony analyses on the alignments were conducted with PHYLIP (version 3.2) (Felsenstein 1989). The DNADIST program of PHYLIP was used to calculate genetic distances according to the Kimura two-parameter (Kimura 1980) and maximum likelihood models of sequence evolution. Trees were constructed from these distances with the NEIGHBOR and FITCH PHYLIP programs to create neighbor-joining trees (Saitou and Nei 1987). For PCR-RFLP analysis, restriction sites were predicted from the sequence data using Webcutter 2.0 (Heiman 1997). Amplified DNA was digested according to manufacturer's recommendations using the restriction enzymes *Nla*III, *Rsa*I, and *Tsp509*I (New England Biolabs), per Cherry et al. (1997). Polymerase chain reaction–RFLP fragments were separated by 10% PAGE (Taylor et al. 1997). Restriction profiles for each enzyme were given letter designations in order of discovery, with the first pattern being designated ''A,'' the second ''B,'' and so on. Haplotypes of each paddlefish were then identified by the combination of letters representing the restriction profiles for each restriction enzyme used. Nested analysis of variance from haplotype frequencies within and among populations was calculated using Analysis of Molecular Variance 1.55 (Excoffier et al. 1992).

Results and Discussion

Seven paddlefish from Nebraska, two from Montana, and one from Louisiana were subjected to DNA sequencing. Relative to the reference sequence (Figure 1), a total of 22 substitutions occurred in the 800 bp of the d-loop region. Nine mitochondrial genotypes were identified among these 10 individuals. Up to five nucleotide site differences were found between the most divergent types. Genetic distance within *P. spathula* ranged from 0.0 to 1.5%, with a mean of 0.7%. Polymerase chain reaction–RFLP analysis revealed six haplotypes (Figure 2), one of which, AAD, was not observed among the 10 individuals sequenced. Polymerase chain reaction–RFLP fragment sizes and restriction enzymes sites are provided in Table 2. Nested analysis of haplotype frequencies revealed that most of the variation occurred within populations and accounted for 94% of the variance when the Galvins Pt. Dam tailwater samples were contrasted with each other. No temporal differentiation (F_{ST} = 0.004) was observed among the 1995, 1998, and 1999 Nebraska samples. The aligned DNA data matrix, including the outgroup taxon, resulted in a total of 875 characters, including gaps. Parsimony analysis of the DNA sequences was unable to reveal any substructuring among the non–PCR-RFLP ''BAC'' haplotypes (Figure 3). The cladogram inferred from the neighbor-joining analysis was nearly identical in topology to the one derived from parsimony analysis. The only differences between the neighbor-joining and the parsimony analyses were DNA sequences NE95B and NE95C, which formed a distinct clade for the neighbor-joining analysis.

We observed nine mitochondrial genotypes from the DNA sequences of 10 individuals and six haplotypes among the 93 paddlefish subjected to PCR-RFLP analysis. All of the observed genotypes and haplotypes were present in the Galvins Pt. Dam tailwater population. This finding, in conjunction with the lack of temporal variation for the 1995, 1998, and 1999 samples from Galvins Pt. Dam tailwater indicates a stable population struc-

```
bp pro-tRNA-2                                                  tRNA-pro          /D-loop
  1 ACCCTTAACT  CCCAAAGCCA  TTAAACTATT  CTCTGAACAA  CACATAAAAT
    Rsa I                   Tsp509 I
 51 GTACTATGTT  TAATCCACAT  TAATTTCTAG  CCACCATAAA  TCATTCACTT
          Tsp 509 I                               Nla III
101 ACTGTATTGA  ATTTTAAATG  CAACAAAAAC  ATAGTATGTT  TAATCCACAT
    Tsp509 I
151 TAATTTTTAC  ACAACATTAA  AGATAACTTG  TATAACCAAC  AATAACCTAC
                Tsp509 I
201 TTTTAAAAAC  ATTAAATAAA  AGTAAGAACC  GACCATCTAT  ATTTCGTT-AA
                                      Tsp 509 I
251 AATATAAAGT  TAATGAGATG  ACGGATAATA  ATTGCAAAGT  TACATAGAAT
301 GAACTATTAC  TGGCATCTGG  TTCCTATTTC  AGGTCCATAA  ATGGTTATTT
                                                   Rsa I
351 CCACATAACT  GAACTATGTC  TGGCATCTGA  TTAATGTTAT  AAGTATTATA
                Nla III                           Tsp 509 I
401 CAATCCGTGA  CCCCACATGC  CAAGAATCTT  GTCAACATCA  GTTAT-TTTTT
                      Rsa I  Nla III               Nla III
451 TAGGTTACCA  TTCACGTACA  TGTAAAAACT  CCTCGAC-ATG  GAAAAATGGA
          Nla   III Tsp509 I
501 AGTGGAACAT  GCTATTTTGT  CCTATAAACA  GCTGAATGAA  TGATTTAATG
551 ACATAAACAT  TATTACACTA  CATTAACCTA  ATACCACGGA  TTATACCTTC
                                      Tsp509 I
601 AACTCTCATA  CTACACCCTG  ATACTTCTTA  AGCCAATTGT  TCTACATTAC
651 ACATTTATAA  CTTTGATGCT  TATTCTCGAC  AAACCCCCTA  CCCCCTTACA
                      Rsa I
701 TTGAACAAGT  TCTTGTACTT  TCCTGTCAAA  CCCCAAAAGC  AGGACTAACT
          Nla   III
751 CGCATCAACA  TGTTTTACCA  TCCCCGACTA  CATAAATATT  TGTATCTATC
                                                   /tRNA-phe
801 ATTATATTCG  TATACATTAT  TACACAATCA  CACAAAATAA  TATAAGCTAG
                phe-tRNA-1
851 TGATGCTTAA  CTAAAGCATA  ACAC
```

FIGURE 1.—Sequence for 875 base pairs of mtDNA from paddlefish sample NE95B. The 22 variable positions among the 10 paddlefish sequences are underlined, and the three restriction enzymes used for PCR-RFLP analysis are listed. Boundaries of the three regions of the genome are indicated, and primer recognition sites are in bold.

ture for this period. Levels of observed genetic variation were similar to those observed by Epifanio et al. (1996). Using total mtDNA RFLP analysis, Epifanio et al. observed four haplotypes from 73 paddlefish collected from nine tributaries (average of eight paddlefish per tributary). No single tributary had all four haplotypes present, and they concluded that paddlefish have low levels of within-population genetic diversity. However, they also observed that their sample sizes were not sufficient to confidently estimate haplotype frequencies within populations. In our study, all of the observed PCR-RFLP haplotypes were present within the Galvins Pt. Dam tailwater population,

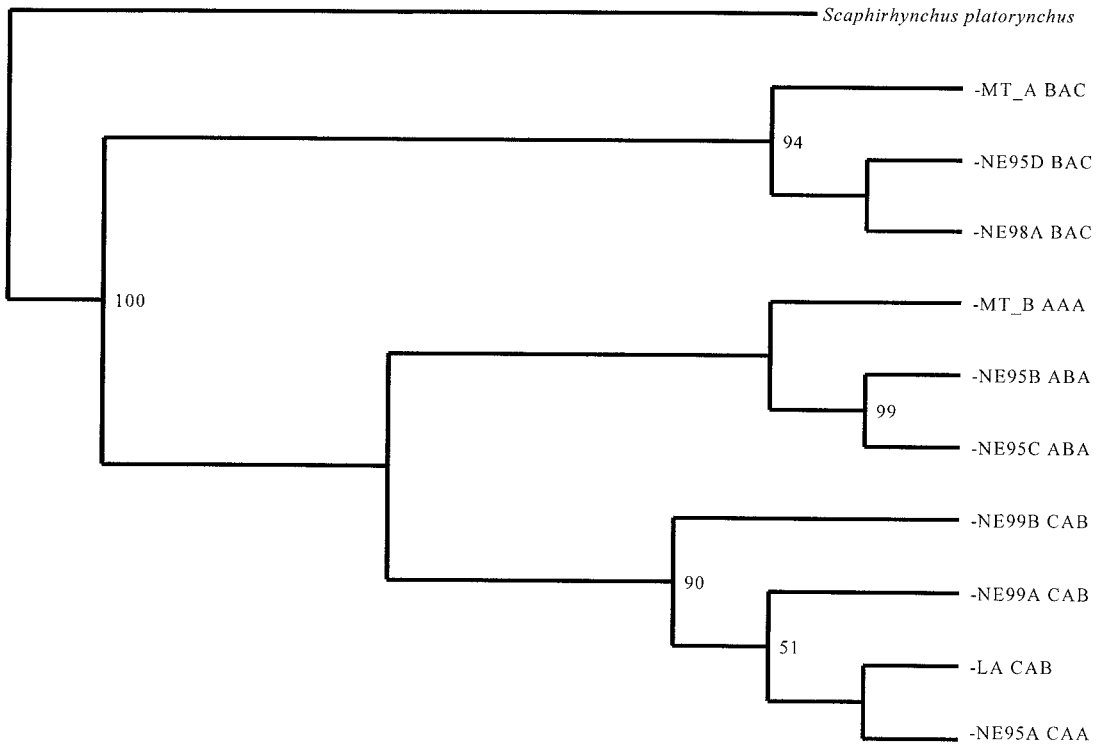

FIGURE 2.—Polymerase chain reaction–RFLP haplotype variation in paddlefish with the restriction enzymes *Nla*III, *Rsa*I, and *Tsp509*I.

a fact that may be attributed to our large sample size from this location. Because our samples from Louisiana and South Dakota were minimal, they can be used only to indicate the presence of the observed haplotypes, and they cannot provide information concerning the absence of other haplotypes.

Polyacrylamide gel electrophoresis allows routine observation of restriction enzyme fragments that are as small as 10 bp, and the thinness of the acrylamide gels relative to agarose gels allows the use of less PCR template for each restriction digest. Finally, two PAGE apparatuses can resolve the fragment patterns of up to four restriction enzymes from 36 samples in 90 min at a significantly lower cost than that associated with agarose gel electrophoresis. Polymerase chain reaction–RFLP based on DNA sequence data represents a robust method for the analysis of numerous paddlefish samples. The method can be useful with DNA ex-

TABLE 2.—Paddlefish PCR-RFLP[a] restriction sites and fragments.

Restriction enzyme and bases	Pattern	Restriction enzyme sites	Fragments (base pairs)
Nla III	A	419 472 490 511 762	419 251 112 53 21 18
CATG/	B	138 419 472 512 763	281 251 138 112 53 40
	C	419 473 512 763	419 251 112 54 39
Rsa I	A	52 394 467 717	342 250 158 73 52
GT/AC	B	52 467 716	415 249 158 52
Tsp509 I	A	71 109 151 279 634	355 240 128 71 42 38
/AATT	B	71 109 151 214 279 634	355 240 71 65 63 42 38
	C	71 109 151 279 439 635	240 196 160 128 71 42 38
	D	71 109 151 279 518 634	240 240 128 116 71 42 38

[a] Polymerase chain reaction–restriction fragment length polymorphism.

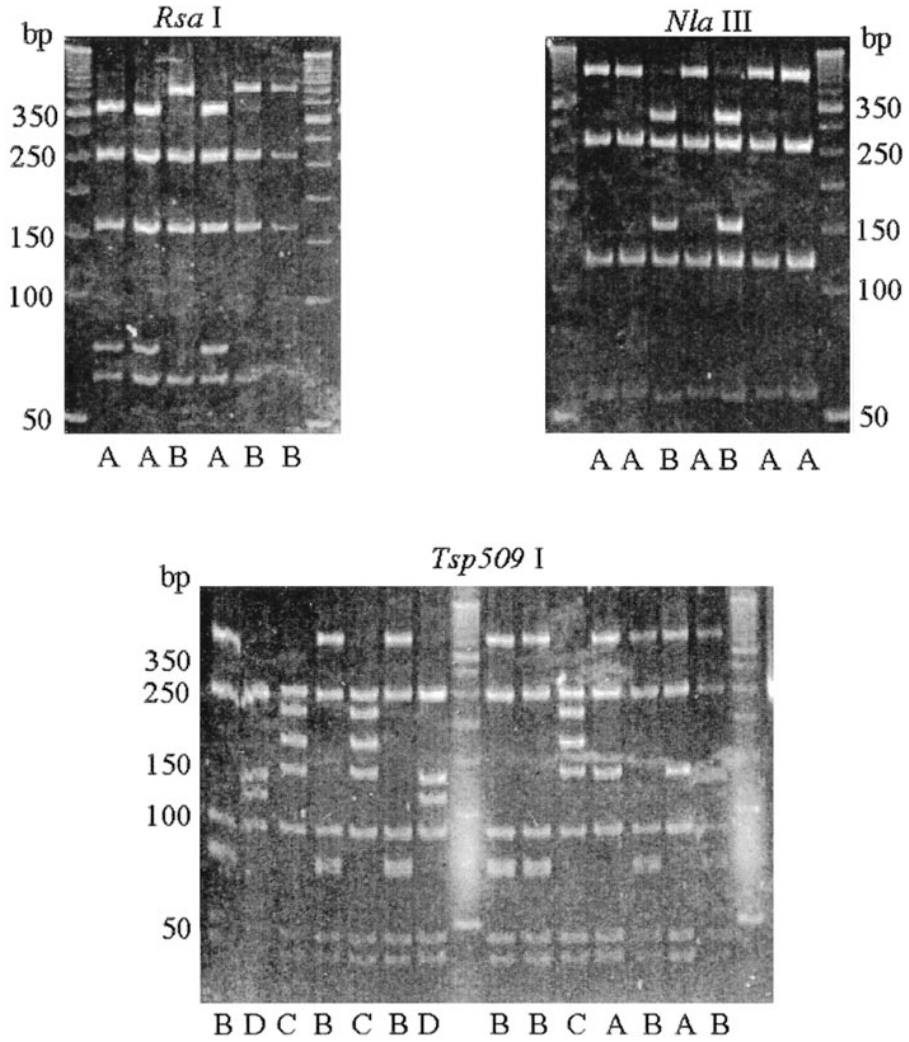

FIGURE 3.—Paddlefish cladogram, derived from parsimony analysis and rooted by the outgroup taxon *Scaphirhynchus platorynchus*. Bootstrap values of greater than 50% and PCR-RFLP haplotypes are provided (restriction enzyme order: *Nla*III, *Rsa*I, and *Tsp*509I).

tracted from fin clippings preserved in ethanol, allowing nondestructive sampling. The ability to use individual eggs will be of benefit for the identification of caviar and for the genetic characterization of stocks before propagation.

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