Length Variation, Heteroplasmy and Sequence Divergence in the Mitochondrial DNA of Four Species of Sturgeon (Acipenser)

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ABSTRACT

The extent of mtDNA length variation and heteroplasmy as well as DNA sequences of the control region and two tRNA genes were determined for four North American sturgeon species: Acipenser transmontanus, A. medirostris, A. fulvescens and A. oxyrhynchus. Across the Continental Divide, a division in the occurrence of length variation and heteroplasmy was observed that was concordant with species biogeography as well as with phylogenies inferred from restriction fragment length polymorphisms (RFLP) of whole mtDNA and pairwise comparisons of unique sequences of the control region. In all species, mtDNA length variation was due to repeated arrays of 78–82-bp sequences each containing a D-loop strand synthesis termination associated sequence (TAS). Individual repeats showed greater sequence conservation within individuals and species rather than between species, which is suggestive of concerted evolution. Differences in the frequencies of multiple copy genomes and heteroplasmy among the four species may be ascribed to differences in the rates of recurrent mutation. A mechanism that may offset the high rate of mutation for increased copy number is suggested on the basis that an increase in the number of functional TAS motifs might reduce the frequency of successfully initiated H-strand replications.

There are two basic types of mitochondrial DNA (mtDNA) sequence polymorphism: nucleotide substitutions caused by point mutations and length variation that can result from varying copy numbers of an oligonucleotide sequence or larger scale genomic duplications. The propagation of any new mutation requires, if only briefly, a transition stage where the multiple forms of the naitochondrial genome coexist within a single individual—the so-called heteroplasmic state. Heteroplasmy for point mutations appear to be rare (Hale and Singh 1986; Moritz et al. 1987) although it must be at least a transient stage in the fixation of alternative sequences in different lineages. The time for the fixation of nucleotide substitutions appears to be rapid and, in cattle, it has been shown to take as little as a single generation (Hauswirth and Lapis 1982; Koehler et al. 1991).

In contrast, heteroplasmy for length variation has been frequently observed (reviewed in Rand 1993). A common type of length variation in vertebrate mtDNA are tandemly repeated sequences in the origin of heavy strand replication and transcription, also known as the control or D-loop region. The latter name describes the resting state trplex structure where a short nascent D-loop strand binds to the light (L) strand thus displacing the heavy (H) strand (reviewed in Clayton 1982). Length variation and heteroplasmic in the control region has led to several proposals for the generation and maintenance of multiple sized mtDNA molecules including nuclear genome-like slipped-mispairing (Estadiadis et al. 1980), intra- and intermolecular recombination (Rand and Harrison 1989) and competitive displacement between D-loop and H-strands (Buroker et al. 1990).

North American sturgeon species of the genus Acipenser provide additional opportunities to infer mechanisms of mtDNA length variation and heteroplasmy. A. transmontanus (white sturgeon) were previously shown to have extensive heteroplasmy due to a varying copy number (1–6) of a perfect tandemly repeated 82-bp element in the D-loop region (Buroker et al. 1990; Brown et al. 1992a). It is widely held that the distinctive eastern and western assemblages of North American fish species resulted from the formation of the Continental Divide, some 10 mya (Smith 1981). Of the sturgeon species examined here, A. medirostris (green sturgeon), like A. transmontanus, are found only in rivers and Pacific coastal waters to the west of the Rocky Mountains while A. fulvescens (lake sturgeon) and A. oxyrhynchus (Atlantic sturgeon) are limited to the Continental interior and Atlantic Coast, respectively. Although recently overfished and their habitats frequently disturbed by dams and pollution, there has been no recorded artificial transplantation of these stur-
geon species. Thus their present day distributions probably reflect natural speciation events. The purpose of this study is to examine mtDNA nucleotide substitutions and length polymorphisms among these closely related but geographically isolated sturgeon species.

**MATERIALS AND METHODS**

Specimen collection and restriction enzyme analysis of mtDNA: Collection of A. transmontanus specimens and mtDNA analysis were described previously (Buroker et al., 1990; Brown et al. 1992a, 1993). A. medirostris specimens were caught in the lower Columbia River, WA. A. oxyrhynchus specimens were collected in the lower St. Lawrence River, Quebec. A. fulvescens specimens originated from two river systems; the Nelson River and Lake Winnipeg, Manitoba, and the upper St. Lawrence River at Lac St. Pierre, Quebec.

mtDNA for RFLP and PCR analyses were purified from liver tissue within 6–72 hr of collection using a modified protocol of Lamsman et al. (1981), which omitted the sucrose gradient step but included an increase in CsCl-ethidium bromide sedimentation time to 60–72 hr. Typical mtDNA yields were 500–500 ng per gram of homogenized tissue. RFLP comparisons were based on digests with 12 restriction enzymes with specificity for six nucleotide (ApaI, BglI, EcoRI, HindIII, PstI and XhoI), multiple six nucleotide (AclI, AvaI, HaeI and HindIII) and multiple five nucleotide (AvaiI) recognition sequences.

Southern blotting and hybridization: For all four sturgeon species, HaeI digests of mtDNA consistently produced a particular DNA fragment, which varied in length from 1.5 to 2.0 kb and, in some individuals, these occurred as multiple bands. To determine whether this fragment spanned the control region, HaeI digested mtDNA was transferred to nitrocellulose filters and probed with the mitochondrial control region cloned earlier (Brown et al., 1990). Three of the D-loop region primers used, I.185, L506 and H740, have been described earlier. New primers are H308 (5′ CAGTTGTAATCCCTACAG 3′) and H482 (5′ AGGAGTTGAGATTCGCT 3′) in the D-loop region; tRNAPhe gene (Gilbert et al., 1988) and Cyb1102 (5′ TGACCGGCTCTAGTAG 3′) in the gene for cytochrome B (Brown et al., 1989).

PCR amplification: PCR primers were designed to amplify D-loop and tRNA genes from all four species with the exception of ADL1PH (5′ AGTAACACAGTCCCTAG 3′), which was specific to A. fulvescens. Control region primers are designated by the letters “L” and “H” (for light and heavy strand, respectively) and by a number corresponding to the position of the primer’s 3′ base in the reference 1.6-kb fragment from A. transmontanus (Buroker et al., 1990). Three of the D-loop region primers used, L185, L506 and H740, have been described earlier. New primers are H308 (5′ CAGTTGTAATCCCTACAG 3′) and H482 (5′ AGGAGTTGAGATTCGCT 3′) in the D-loop region; tRNAPhe gene (Gilbert et al., 1988) and Cyb1102 (5′ TGACCGGCTCTAGTAG 3′) in the gene for cytochrome B (Brown et al., 1989).

PCR amplification, DNA fragment isolation and sequencing protocols have been previously described (Brown et al., 1993). Occasionally, PCR amplified DNA fragments extracted from agarose gels were blunt-end ligated into the plasmid vector pUC19 (Messing and Vieira, 1982). Selected recombinants were then sequenced using either universal sequencing primers or internal primers to the cloned fragment. A minimum of three plasmid isolates of each target clone were sequenced.

**DNA sequence analysis**: Nucleotide substitutions per site (d) between pairs of mtDNA genotypes were estimated from RFLP data using the maximum likelihood method of Nei and Tajima (1983). A distance tree of percent sequence divergence (d) was constructed using the unweighted pair-group method with arithmetic mean (UPGMA) and standard errors about the nodes were calculated (Nei et al., 1985).

**Table 1**: Pairwise estimates of sequence divergence among four sturgeon species, genus Acipenser

<table>
<thead>
<tr>
<th>Species</th>
<th>A. transmontanus</th>
<th>A. medirostris</th>
<th>A. fulvescens</th>
<th>A. oxyrhynchus</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. transmontanus</td>
<td>—</td>
<td>14.07 (19.70)</td>
<td>29.65 (16.69)</td>
<td>32.49 (23.89)</td>
</tr>
<tr>
<td>A. medirostris</td>
<td>4.14 ± 0.96</td>
<td>—</td>
<td>29.31 (15.72)</td>
<td>32.70 (16.95)</td>
</tr>
<tr>
<td>A. fulvescens</td>
<td>6.65 ± 1.29</td>
<td>8.27 ± 1.50</td>
<td>—</td>
<td>38.30 (18.12)</td>
</tr>
<tr>
<td>A. oxyrhynchus</td>
<td>7.82 ± 1.59</td>
<td>8.49 ± 1.69</td>
<td>9.07 ± 1.72</td>
<td>—</td>
</tr>
</tbody>
</table>

The lower diagonal presents percent sequence differences and standard deviations estimated from RFLP analysis of whole mtDNA using the maximum likelihood method of Nei and Tajima (1983). The upper diagonal shows pairwise estimates of the expected number of nucleotide substitutions per 100 sites between non-repeated portions of the control region using Kimura’s (1980) correction for multiple substitutions. In parenthesis, similar estimates are given between single copies of central repeats.
DNA sequences were aligned by eye using the program ESEE v3.0 (CABOT and BECKENBACH 1989). Distance estimates and neighbor-joining trees were determined using MEGA v1.01 software (KUMAR et al. 1998) as well as the PHYLIP programs DNADIST, SEQBOOT and NEIGHBOR (FELSENSTEIN 1993). Pairwise sequence differences, omitting indels, were estimated using the two parameter model for nucleotide substitutions of KIMURA (1980). Confidence intervals for internal branching points in neighbor-joining trees were estimated by 500 bootstrap replications as implemented by MEGA.

Thermal stabilities of repeat secondary structures were determined for one or more repeat elements using the PCFOLD algorithm of ZUKER and STIEGLER (1981). A search for secondary structures that would base-pair to the limits of repeat elements was done by ascertaining the thermal stability of duplex structures that were offset by one nucleotide per determination and forced to fold to the end of the fragment.

RESULTS

Restriction enzyme analysis: The 12 restriction enzymes detected between 40 (A. oxyrhynchus) and 59 (A. fulvescens) sites per mtDNA genome. Restriction enzyme maps and sequence alignments available upon request from J. R. BROWN [GenBank Accession Nos U30601 (Am5), U30728 (Am12), U32308 (Ao8), and U32309 (Af14)]. The size range, between 16.1 to 16.5 kb, of A. medirostris, A. fulvescens and A. oxyrhynchus mtDNA were similar to that reported for A. transmontanus (BROWN et al. 1992a,b). Sequence divergence estimates between four sturgeon species (Table 1) were at least fourfold greater than intraspecific differences (BROWN et al. 1992b). A. transmontanus and A. medirostris, found west of the Continental Divide, were the most closely related species pair (4.14% sequence difference). The two eastern species, A. fulvescens and A. oxyrhynchus, were highly diverged (6.65–8.49% difference) from the western species pair although A. fulvescens was marginally more closely related. The separation of species pairs was well supported by standard error and bootstrap statistical tests of the respective distance (UPGMA) and parsimony (Wagner) phylogenies (Figure 1).

RFLP data suggested that genetic diversity within each species is low. Out of 10 A. oxyrhynchus, nine individuals had identical restriction enzyme site maps while the remaining individual differed by only a single AvaII site (0.174% difference). Among 12 A. fulvescens individuals, two closely related genotypes (0.314% difference) were detected by HindII and AvaII restriction site polymorphisms. Six and four A. fulvescens individuals from the Nelson River, Manitoba, and Lac St. Pierre, Quebec, respectively, shared one genotype while two individuals from the Nelson River had a second genotype.

mtDNA length variation: In Southern blots of HaeIII digested mtDNA, the cloned control region of A. transmontanus (BUROKER et al. 1990) strongly hybridized to fragments varying in size from 1.5 to 2.0 kb in all four species, an indication that these variable length fragments did indeed span the D-loop region (Figure 2). Sequence analysis later revealed that one HaeIII site was located ~74 bases upstream (on the light strand) of the control region in the gene for tRNAThr. The second HaeIII site was not found in the sequenced 3' terminus of the control region and, therefore, must be located further downstream.

Previously, it was shown that length variation of the A. transmontanus D-loop was due to varying numbers, from one to six copies, of an 82-bp sequence repeated in tandem (BUROKER et al. 1990). These repeated arrays were followed by a degenerate or partial copy of the repeat. In the present study, Southern blot analysis revealed that D-loop length variants of the new sturgeon species closely corresponded to those discrete size classes observed in A. transmontanus (Figure 2). This permitted a standardized ranking of mtDNA size variants across species relative to the observed A. transmontanus repeats (Table 2). Chi-square analysis revealed a highly significant difference between the frequencies of mtDNA size classes among all four species ($\chi^2 = 43.77, P < 0.01$). However, when the A. oxyrhynchus data were removed, no significant differences were observed among the remaining three species ($\chi^2 = 7.90, P = 0.61$).

The incidence of heteroplasmy was strikingly different between west and east species clades. About 42% of 174 sampled A. transmontanus individuals were heteroplasmic for at least two different mtDNA size variants.
one A. oxyrhynchus individual had a larger D-loop fragment that roughly corresponded to an A. transmontanus D-loop with two repeats.

The mean genetic diversity within individuals ($K_i$) of the two western species were not significantly different (Student’s $t = -0.782$; d.f. = 183; $P = 0.44$; Table 2). In the three species with extensive mtDNA length polymorphism, most of the genetic diversity could be attributed to variation between, rather than within, individuals ($K_i$ and $G_{vp}$).

**DNA sequences of tRNA genes:** The DNA sequences of the genes for tRNA$^{\text{Thr}}$ and tRNA$^{\text{Pro}}$ of A. medirostris, A. fulvescens and A. oxyrhynchus differed little from those previously determined for A. transmontanus (Gilbert et al. 1988). The only mutations were a single substitution in the three nucleotide intergenic region of A. fulvescens and a single transitional substitution and a single base insertion in the tRNA$^{\text{Thr}}$ and tRNA$^{\text{Pro}}$ genes, respectively, of A. oxyrhynchus. None of these changes altered the previously proposed secondary structures for these tRNAs.

**The control region:** Entire and partial control regions were sequenced from selected individuals with varying numbers of putative repeats (Table 3). General organizations of control regions from the three additional sturgeon species are similar to that previously reported for A. transmontanus (Figure 3; Buroker et al. 1990).

The overall architecture of sturgeon species' control regions closely resembles that of higher vertebrates with respect to the position of conserved sequence blocks or CSBs that have been implicated in the initiation of transcription and heavy strand replication (reviewed in Clayton 1982). The repeat region, responsible for both length variation and heteroplasmy is near the end of the D-loop region, separated by two or three nucleotides from the tRNA$^{\text{Pro}}$ genes. These repeats consistently have D-loop strand termination associated sequences or TAS motifs and an additional, divergent TAS-like block can be identified in the tRNA$^{\text{Pro}}$ gene. Separating the repetitive region and CSBs is a region of unique

### Table 2

<table>
<thead>
<tr>
<th>Species</th>
<th>N</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>$K_i$</th>
<th>$G_{vp}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. transmontanus</td>
<td>174</td>
<td>0.273</td>
<td>0.251</td>
<td>0.283</td>
<td>0.144</td>
<td>0.051</td>
<td>0.018</td>
<td>0.185 (±0.018)</td>
<td>0.762 (±0.758)</td>
</tr>
<tr>
<td>A. medirostris</td>
<td>10</td>
<td>0.027</td>
<td>0.306</td>
<td>0.476</td>
<td>0.192</td>
<td>0.000</td>
<td>0.000</td>
<td>0.247 (±0.009)</td>
<td>0.642 (±0.615)</td>
</tr>
<tr>
<td>A. fulvescens</td>
<td>21</td>
<td>0.381</td>
<td>0.143</td>
<td>0.238</td>
<td>0.190</td>
<td>0.048</td>
<td>0.000</td>
<td>0.000 (±0.000)</td>
<td>0.739 (±0.10)</td>
</tr>
<tr>
<td>A. oxyrhynchus</td>
<td>19</td>
<td>0.947</td>
<td>0.053</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000 (±0.000)</td>
<td>0.000 (±1.0)</td>
</tr>
</tbody>
</table>

Size variants in all species were categorized according to distance travelled on a 1.2% agarose gel relative to mtDNA from a highly heteroplasmic A. transmontanus individual (see Materials and Methods). $K_i$ is the mean of the values, $K_i = 1 - \sum x_i^2$, where $x_i$ is the proportion of the $i^{th}$ copy number in each fish. The standard errors of $K_i$ are in parenthesis. $K = 1 - \sum x_i^n$, where $x_i$ is the frequency of the $i^{th}$ size variant in each species. $G_{vp} = (K - K_i)/K_i$ is the size diversity occurring between, rather than within, fish.
sequence, segments of which were polymorphic within a species (Figure 3).

Pairwise sequence comparisons of these nonrepeated or unique D-loop segments confirmed the species relationships suggested by RFLP analysis of whole mtDNA (Table 1). Neighbor-joining distance trees supported the separation of western and eastern species pairs in nearly 99% of 500 bootstrap replicates. Interspecific nucleotide substitution rates of unique D-loop sequences were estimated to be about four to five times greater than the average rates of the entire mtDNA genome (as estimated from RFLP comparisons), which is consistent with early calculations of intraspecific relative mutation rates for *A. transmontanus* (Brown et al. 1993) and human (Aquadro and Greenberg 1983) hypervariable D-loop sequences.

Previous pairwise comparisons of *A. transmontanus* hypervariable sequences from 27 individuals revealed a

![FIGURE 3.—Schematic diagram of aligned light strand sequences of *Acipenser*. An ~1000–1100-bp segment extending from the 3' end of the cytochrome b (cytoB) gene, through the genes for tRNA\(^{Thr}\) (tThr) and tRNA\(^{Pro}\) (tPro), to the conserved sequence blocks (CSB) located on the 3' end of the control region were sequenced from the sturgeon species: *A. transmontanus* (designated *A. trans.*; Buroker et al. 1990), *A. mediostris* (*A. medi.*), *A. fulvescens* (*A. fulv.*), and *A. oxyrhynchus* (*A. oxyr.*). The relative positions of tandem D-loop repeats (labeled 1–5) are shown as well as the degree of sequence conservation among repeats within each species array relative to the central repeat (sequences and position of TAS motifs shown above). On the basis of intraspecific pairwise comparisons to the central repeat, the flanking repeats differed by either 0% (white fill), <5% (cross-hatch); between 20 and 30% (vertical lines) or between 30 and 40% (black fill) nonidentical nucleotides. The number of repeats shown for each species reflects the maximum number sequenced within any individual rather than the maximum number in any species detected by Southern blot hybridizations. In the case of *A. oxyrhynchus*, the repeats are displaced from the tPro gene by a segment, RTX, of limited identity with the repeat elements.  

![Central Repeats](image-url)

**TABLE 3**

*Acipenser* control region clones and repeat copy numbers

<table>
<thead>
<tr>
<th>Species</th>
<th>Clone label</th>
<th>Repeat copy no.</th>
<th>No. of exact copies within an individual</th>
<th>No. of exact copies within species</th>
<th>Repeat length</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. transmontanus</em></td>
<td>At1.9</td>
<td>4 + 1</td>
<td>4</td>
<td>5</td>
<td>82 bp</td>
</tr>
<tr>
<td></td>
<td>At1.6</td>
<td>1 + 1</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. mediostris</em></td>
<td>Am5</td>
<td>2 + 1</td>
<td>2</td>
<td>6</td>
<td>78 bp</td>
</tr>
<tr>
<td></td>
<td>Am6</td>
<td>2 + 1</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Am12</td>
<td>3 + 1</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. fulvescens</em></td>
<td>Af14</td>
<td>3 + 1</td>
<td>2</td>
<td>2</td>
<td>82 bp</td>
</tr>
<tr>
<td></td>
<td>Af17(^a)</td>
<td>2 + 1</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. oxyrhynchus</em></td>
<td>Ao8</td>
<td>2 + 1</td>
<td>0</td>
<td>2</td>
<td>78–79 bp</td>
</tr>
<tr>
<td></td>
<td>Ao12</td>
<td>1 + 1</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ao17(^a)</td>
<td>1 + 1</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Repeat number is given as \(n + 1\), where \(n\) is the number of nearly identical repeats and " + 1" indicates a 3' terminal partial repeat.

\(^b\) Total number of sequenced repeats identical within a species.

\(^c\) Buroker et al. 1990.

\(^d\) Partial loop sequences.
fuluescens

Individuals and within each species but less so between species (BROWN et al. 1993). Low levels of sequence variation were also observed for A. mediorestris (0.0; N = 3), A. fuluescens (0.018; N = 2), and A. oxyrhynchus (0.002–0.008; N = 3).

Repeateed elements: mtDNA length variation could be attributed to different copy numbers of tandemly repeated sequences in the D-loop region (Figure 3). Each species had at least one complete copy of their respective repeated element followed by a 3’ terminal degenerate repeat (Figure 3 and Table 3). Depending on the species, individual repeats were 78-82 bp in length with a putative TAS motif similarly located in the respective repeated module (Figure 3).

The central repeats were well-conserved within individuals and within each species but less so between species (Table 1; Figure 3). The repeated elements in A. fuluescens most closely resembled those of A. transmontanus (BÜRÖKER et al. 1990) although the range of distances among all species was quite narrow (0.157–0.239 corrected nucleotide substitutions per site). Overall, the 5’ end of the repeats showed greater sequence conservation than the 3’ end (Figures 3 and 4).

Intraspecific and intraindividual conservation of repeat sequences were greatest in A. transmontanus (BÜRÖKER et al. 1990) and A. mediorestris. In the latter species, the basic repeat length was 78 bp. Of the A. mediorestris D-loops sequenced, two individuals (Am5 and Am6) each had two perfect repeats while a third (Am12) had three repeats. mtDNA from Am5 and Am6 was also identical with respect to comparisons of RFLPs and unique D-loop regions. The first two repeats in all three individuals were identical while the third repeat in individual Am12 differed from the perfect repeats at three nucleotide positions (Figure 4).

The two A. fuluescens individuals (Afl4 and Afl7) examined had four and two repeats in their respective control regions with each repeat being 82 bp in length. The second repeat element was only partially sequenced for Afl7 (Figure 4). In contrast to A. transmontanus and A. mediorestris, the second and third repeats in A. fuluescens were identical while the first repeat differed by two C-T transitions. The first repeat of Afl7 differed by two C-T transitions.

Relative to the other three sturgeon species, length variation in A. oxyrhynchus mtDNA is rare. A larger D-loop fragment was detected in only one of the 19 individuals investigated. Of the three A. oxyrhynchus individuals sequenced, two (A012 and Ao17) had a single repeat element, 78 bp in length, the other (Ao8) had two complete copies (CR and RT3; Figure 4). The first repeats in individuals Ao8 and Ao12 were identical while that of Ao17 differed by single base insertion (Fig-
junction regions are themselves five nucleotide palindromes. In all four species, thermal stabilities for potential hairpins of repeats that could form a hairpin with a terminal stem are also lower than those calculated previously for the repeat unit that could form a secondary structure with a terminal hairpin stem. In the remaining species, the segments that could form a hairpin with a terminal stem were all smaller than the estimated repeat lengths. Although A. medirostris 78 bp long repeats will fold to form secondary structures, they will not form hairpin stems base-paired exactly to the 5' and 3' terminus of the repeat units. For terminus base-pairing to occur, the element is 77 bp long and the next element is removed by one nucleotide (C) for each repeat. The estimated thermal stabilities (Table 4) for the A. medirostris repeats are somewhat lower than those previously reported for A. transmontanus repeats (Buroker et al. 1990).

In A. fulvescens, only one terminally base-paired 76-bp hairpin structure near the repeat length could be formed, from nucleotide position 37-112 of the D-loop region. In A. oxyrhynchus, a terminally base-paired stem segment 77 bp in length is possible from base pair 120-200 and base pair 202-283 of the D-loop region. These junction regions are themselves five nucleotide palindromes. Thermal stabilities for potential hairpins of repeat elements found in A. oxyrhynchus and A. fulvescens are also lower than those calculated previously for A. transmontanus (Table 4).

### Table 4

<table>
<thead>
<tr>
<th>Species</th>
<th>Number of repeats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>A. transmontanus&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-14.1</td>
</tr>
<tr>
<td>A. medirostris&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-8.5</td>
</tr>
<tr>
<td>A. fulvescens&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-12.4</td>
</tr>
<tr>
<td>A. oxyrhynchus&lt;sup&gt;d&lt;/sup&gt;</td>
<td>-6.8</td>
</tr>
</tbody>
</table>

Thermal stability was kcal M<sup>-1</sup>
<sup>a</sup>78-nt element repeats starting at bp 5 in Figure 4.
<sup>b</sup>82-nt element repeats starting at bp 1 in Figure 4.
<sup>c</sup>79-nt element repeats starting at bp 5 in Figure 4.
<sup>d</sup>82-nt element repeats starting at bp 5 in Figure 4.

**DISCUSSION**

We determined the structure of the D-loop in four North American species of sturgeon. In all four species, multiple copies of a 78–82 basepair segment bearing the termination associated sequences (TAS) were found. Three different structural patterns were observed in these species. In the two West Coast species, repeat copy numbers varied both between and within individuals. In contrast, no heteroplasmy was found in the Eastern species. A. fulvescens individuals were fixed for varying numbers of repeats, while all but one A. oxyrhynchus were fixed for a single copy of this segment. To attempt to understand these patterns, we first examine the phylogenetic relationships of the species.

**Sturgeon phylogeny**: The phylogeny of the four Acipenser species, as inferred from either whole mtDNA RFLP or unique control region sequence data, clearly shows distinctive species assemblages on opposite sides of the Continental Divide. The Pacific Coast species, A. transmontanus and A. medirostris, are more closely related to each other than to either species living east of the Divide, A. fulvescens and A. oxyrhynchus.

A. transmontanus and A. medirostris apparently diverged from each other well after the genetic isolation of western fish fauna. The present-day distributions of these species overlap. A. medirostris inhabits marine coastal waters and migrates short distances inland to spawn in freshwater while A. transmontanus has a similar marine distribution but is often found further inland in lakes and rivers along the western slope of the Continental Divide (Scott and Grossman 1973). The separation of A. oxyrhynchus from the three other species was probably the most ancient divergence event and may have occurred in the Mississippi Basin. The contemporary distribution of A. fulvescens includes the middle to upper areas of the Mississippi drainage while A. oxyrhynchus are found in the lower river and coastal waters (Swift et al. 1986). The long term ecological stability of the Mississippi River throughout the Cenozoic likely facilitated a broadening of species diversity, which, perhaps, included the emergence of new species of sturgeon (Smith 1981; Robison 1986).

**Nucleotide substitution rates**: Among mammalian mtDNAs, the generalized rate of nucleotide substitution has been estimated to be ~2% per million years (reviewed in Brown 1985; Wilson et al. 1985). Application of this rate to the present RFLP data would suggest that A. fulvescens diverged from A. transmontanus and A. medirostris ~3.5 mya. This estimated time of divergence is much later than speculation events that are postulated to have occurred as a result of transcontinental uplifting and block-faulting during the mid- to late Miocene, some 7–12 mya (Cavender 1986).

In addition to the RFLP data (sampling the entire mitochondrial genome), we can examine the evolutionary rate of the D-loop sequence. Previous intraspecific analysis of A. transmontanus mtDNA estimated the nucleotide substitution rate of unique D-loop sequences to be four- to fivefold higher than that observed in the mitochondrial genome as a whole (Brown et al. 1993).
Present pairwise comparisons among sturgeon species confirm this elevated relative mutation rate and comparable estimates have been made for human D-loops (Aquadro and Greenberg 1983). Pairwise comparisons among human hypervariable D-loop sequences suggest an absolute mutation rate of ~8% per million years (Viligant et al. 1989). Provisional application of this rate to sturgeon suggests that the divergence of A. transmontanus and A. medirostris from A. fulvescens occurred ~5.7 mya while the subsequent A. transmontanus and A. medirostris split happened ~2.2 mya. These estimated times of divergence are similar to those derived from RFLP analysis and are not compatible with Miocene speciation events.

Interspecific comparisons based on a Miocene separation of East and West Coast species suggest a two- to fourfold lower mutation rate in sturgeon relative to mammalian mtDNA. A substantially slower rate of nucleotide substitution has been observed in a number of large, long-lived ectotherms (Martin et al. 1992; Avise et al. 1992; Martin and Palumbi 1993). If we assume that the Western clade has been isolated for 10 mya, we estimate a divergence time of ~6 mya for A. transmontanus and A. medirostris.

Concerted evolution: While A. medirostris and A. transmontanus individuals share patterns of mtDNA size variation and heteroplasmcy, the actual nucleotide composition of repeat units differed between species. In two A. medirostris individuals with three and four repeats, the initial (5' end) two and three repeats, respectively, were nearly identical both within and between individuals. A similar intraspecific pattern of sequence conservation of repeat elements was reported for A. transmontanus and A. medirostris.

Secondary structure analysis suggests that 78-bp repeats in A. medirostris might be capable of forming stemloop structures comparable with those proposed for 82-bp repeats in A. transmontanus. The illegitimate elongation model predicts that an array of consecutive repeats, the central repeats should evolve in a concerted manner (Buroker et al. 1990). Nucleotide sequences of the repeats may vary between distinctly related genotypes (or species) but within a genotype, repeats should be perfectly conserved. Apparently, such is the case for repeats in A. medirostris and A. transmontanus. The high level of sequence identity among central repeats both within and between individuals of each species, suggests the existence of some on-going process that serves to homogenize the sequences of central, adjacent repeats.

In A. fulvescens, the nucleotide compositions of the second and third repeats were identical within a single individual but not between individuals. The first repeat was slightly different in comparison to subsequent repeats within the same array as well as to the initial repeat in another individual. A. oxyrhynchus individuals were consistently fixed for single repeats with the exception of an individual with two, nonidentical copies. The occurrence of nucleotide substitutions and single indels among repeat elements, suggests that sequence homogenization processes in A. fulvescens and A. oxyrhynchus are not acting as strongly as those present in heteroplasmic sturgeon species.

Several reports of heteroplasmy involving tandem repetitive sequences in the D-loop region indicate repeat segments can fold into putative stable structures, either singly or as multiples, leading to the addition or deletion of repeat elements (Buroker et al. 1990; Wilkinson and Chapman 1991; Arnason and Rand 1992; Stewart and Baker 1994). It must be noted, however, that models for the prediction of stable DNA secondary structure, and perhaps more importantly tertiary interactions, are inexact.

mtDNA size variation and heteroplasmcy: A number of authors have suggested that heteroplasmy involving tandem repeats is dynamically maintained by high mutation rate, offsetting the effects of rapid segregation (Rand and Harrison 1987; Buroker et al. 1990). As Clark (1988) noted, relatively subtle differences in mutation rate may determine whether a system is tipped in favor of mutations leading to heteroplasmy, or segregational elimination of heteroplasmy. As a byproduct of high levels of mutation between different size classes, we would expect concerted evolution to maintain sequence identity among the repeats in any mitochondrial lineage.

Length heteroplasmy as seen in mitochondrial D-loops evidently requires several features: the presence of tandem repeats, a high degree of sequence identity among repeats, and the capability to form secondary structure when single stranded. Loss of an ancestral heteroplasmic condition could presumably occur by any of three mechanisms: segregational loss of tandem repeats in lineages and species, point mutations in repeat elements, reducing the ability for slipped-strand pairing, and point mutations reducing the secondary structural characteristics. Gain of heteroplasmy, either from D-loop structures lacking repeats, or from a variable but fixed multicopy state as observed in A. fulvescens, would appear to be less likely.

The results of this study shows that two species that diverged ~6 mya share the multicopy heteroplasmic state. We also find that tandem repeats, either fixed or heteroplasmic, are present in all four taxa. The repeats are very similar in both length and sequence, suggesting that at least a multicopy D-loop structure was present in an ancestor common to all four species perhaps ~12 mya. We suggest that the ancestral condition was heteroplasmic as well, and that heteroplasmy was secondarily lost independently in both eastern species. Under this hypothesis, A. fulvescens, lost the heteroplasmic condition but not the high frequency multicopy state. Most A. oxyrhynchus individuals bear only a single copy. Loss of heteroplasmy in this species could have occurred by any of the three mechanisms noted above. If the initial
step was fixation of a variable multicopy condition, then we must assume that multiple copies were lost through some other mechanism.

It should be noted that we cannot rule out the alternative that heteroplasm was gained in the western species, either in the common ancestor or as recent independent events in each species. But the phylogenetic evidence indicates that the origin of the tandem repeat array is much older. When the array first arose, at least two of the three requirements for heteroplasm would be met: multiple tandem copies and sequence identity of those copies.

**Mutation and the distribution of copy number:** It is clear that mutation rates altering copy number play an important role in the maintenance of heteroplasm and the maintenance of sequence identity among repeats. At least three mutation rates must be considered: the mutation rate to increased copy number when only a single copy is present, the mutation rate to decreased copy number when multiple tandem repeats are present, and the mutation rate to increased copy number when multiple copy numbers are present. The latter two parameters are believed to be quite high in species with high levels of length heteroplasm (RAND and HARRISON 1989; BUROKER et al. 1990). Mutations from a single copy are generally assumed to be rare.

Mutation rate to increase or decrease copy number from a multicopy state must be considered separately. Under the illegitimate elongation model of BUROKER et al. (1990), only the displaced strand is single stranded. If the D-loop strand is displaced, it will be free to form hairpins. Folding of the D-loop strand is an essential step leading to increased copy number in that model. Under recombination models, such as that proposed by RAND and HARRISON (1989), the distinction between the mechanisms for increased and decreased copy number is even more pronounced.

The relative magnitudes of these mutation parameters may play a major role in determining the distributions of copy number in these organisms. We have previously analyzed these distributions in *A. transmontanus* (BROWN et al. 1992a). The distributions of length variation in all four species is shown in Figure 5. For two of the species, *A. transmontanus* and *A. fulvescens*, the distributions are evidently compounded with a mode at one copy, and a second mode at three repeats. The mode at one copy is easily explained, both as a boundary condition, and as a near-absorbing state because we assume that mutations from a single copy structure are infrequent. The mode at three repeats is more interesting. If the mutation rate to decreased copy number exceeds the rate to increased copy number, mutation pressure should eliminate multiple copy genomes. If the rate to increase copy number exceeds the rate of mutation to lower copy number, then evolution should lead to large numbers of copies, unless opposed by other factors. If the two rates are identical, then other factors will determine the distribution.

Two forces opposing the accumulation of large numbers of repeats have been proposed: selection (reduced fitness at the organismal level) and a replication race during the buildup of mitochondria during oogenesis (RAND 1993). It is not clear how much effect either of these two mechanisms would have on a short, evidently noncoding segment in an 16-kb genome. We offer an alternative mechanism that might apply when the repeated segment includes the TAS motif.

**A TAS-based replication model:** MADSEN et al. (1993), using an *in organello* methylation protection assay, discovered an approximately 48-kD trans-acting protein that binds to a conserved TAS-like sequence in bovine mtDNA control regions. Although multiple TAS-like sequences were identified on the basis of sequence similarity, protein-binding was shown to be specific to only one type of TAS motif. It was suggested that the synthesis of D-loop strands could be arrested by the formation of a single TAS-protein complex although the involvement of additional factors in the initiation of H-strand synthesis cannot be ruled out. H-strand replication appears to be tightly regulated since, although D-loop strand initiation and termination continuously occur, only ~0.05 of the initiated D-loop strands lead to completed cycles of H-strand synthesis (BOGENHAGEN and CLAYTON 1978).

If TAS motifs are involved in the negative regulation of D-loop strand synthesis, then a mechanistic explanation for the replicative advantage of smaller mitochondrial genomes can be postulated. Provided that quantities of the TAS-binding trans-acting factor are not saturating, there would be a 0.05 probability that, at any one moment, a single TAS block will not be bound to a protein thus allowing H-strand synthesis to proceed. If more than one functional TAS-like sequence were present, as may be the case for multiple repeated
elements in sturgeon, then the successful initiation of
H-strand synthesis would depend upon the probability that
all TAS sequences were simultaneously unbound, defined here as p(TAS). This probability could be
described by the simple exponential function p(TAS) = 
(0.05)n where n is the number of boundable TAS se-
quences. This is a very simplistic model since the in vivo
frequency of TAS-protein complex assembly and the successful completion of H-strand synthesis are likely
mediated by additional factors (CLAYTON 1991; MADSEN
et al. 1993).

However, this model of TAS-based regulation of H-
strand synthesis can be reconciled with some pan-spe-
cies observations on the structural arrangements and
frequency distributions of repeat sequences. First, the
TAS-limitation model predicts an exponential decline in
successful H-strand replication with increasing repeat
copy number. The resulting distribution would be
skewed toward genomes with fewer repeats, which is
consistent with observations in sturgeon (BROWN et al.
1992a) and several other species (see figure 4 in RAND
1993). The second prediction is that the replication of
genomes with a large number of repeats (say more than
seven copies) will be rare events. Accordingly, for verte-
brate species in which mtDNA length variation consists of
short (<200 bp) tandem repeats with embedded TAS-like
motifs, the number of repeats in an array ranges from one to eight copies (RAND 1993). While
some species, such as sturgeon, appear to require only
a single repeat for a functional control region, other
vertebrates, including treefrog (YANG et al. 1994),
shrews (STEWART and BAKER 1994) and bats (WILKIN-
sion and CHAPMAN 1991), have arrays with a minimum of four to five repeats, each with an embedded TAS
motif. The higher minimal number of repeats in these
species might be due to yet uncharacterized TAS-pro-
tein binding requirements for specific DNA strand con-
figurations, such as stable secondary structures involv-
ing multiple repeats.

Figure 5 shows the frequency of repeats in different
sturgeon species. While the extremes of this distribu-
tion closely correspond to the observed frequencies
of genomes with either a single repeat or four or more
repeats, there is a lack of correspondence between the
predicted and observed frequencies of two and three
repeats. Earlier, it was suggested that this distribution
reflects recurrent mutation for higher repeat copy num-
ber partially offsetting selection for smaller genome
size. Accordingly, the frequency of mutation for increas-
ing size might be sufficient to overcome the frequency
of assembling protein-DNA complexes at TAS motifs
responsible for D-loop termination. Alternatively, sec-
ondary structural formations involving a certain num-
ber of repeats might be more predisposed to promote
D-loop strand elongation than structures formed from
either fewer or more repeats.

Similarly skewed distributions have been observed in
invertebrates such as crickets (RAND and HARRISON
1989) and Drosophila (HALE and SINGH 1986). The
repeats in crickets have been localized to the A-T rich
region, which has been implicated in insect mtDNA
replication. However, the regulation of H-strand synthe-
sis in invertebrates is poorly understood and sequences
motifs homologous to TAS and CSB sequences in verte-
brate control regions have not been identified (re-
viewed in CLAYTON 1991). Therefore, the present TAS-
limitation model cannot be convincingly extended to
invertebrate mtDNA at this time.

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