Chapter 4
Molecular Markers and the Study of Phylogeny and Genetic Diversity in North American Sturgeons and Paddlefish

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Abstract For a number of reasons, including their threatened or endangered status and importance in caviar production, much effort has been and is being expended worldwide on the study of the genetic variation of sturgeons and paddlefish. Presented here is a review of the genetic studies that have been conducted on the ten North American acipenseriform taxa and the types of molecular markers that have been used in these studies. The results that have been obtained from this research are invaluable for guiding conservation efforts by increasing our understanding of the relationships among species within the group, identifying intraspecific population structure and shedding light on acipenseriform life history traits.

Keywords Acipenseriformes, North America, sturgeon, paddlefish, nuclear DNA, mtDNA, molecular markers, phylogeny, population genetics

4.1 Introduction

Currently, most members of the order Acipenseriformes worldwide are considered threatened, endangered or, in some cases, even extinct. As a result, these fish have become the focus of research on their general biology, aimed at survival and recovery, including the study of their conservation and genetics. Within the area of conservation genetics, molecular markers play an important role in helping us understand sturgeons and paddlefish at many different levels. An appreciation of evolutionary relationships obtained from species divergence of genes among acipenseriform taxa can guide decisions about the focus of conservation efforts, while information about intraspecific genetic variability can identify populations that

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should be managed as distinct stocks or provide information about populations that may be suitable genetic sources for restocking depauperate areas. The aim of this paper is to review the types of genetic studies that have been conducted on the ten acipenseriform taxa found in North America, describing the kinds of molecular markers that have been used in these studies and the results that have been obtained by using genetic approaches to study populations.

4.2 Rates of Molecular Evolution in the Acipenseriformes

Following genetic and karyotypic studies, it was proposed that sturgeons and paddlefish have a reduced rate of molecular evolution (Birstein and Vasiliev, 1987; Brown et al., 1996; see Krieger and Fuerst, 2002a for a review). Krieger and Fuerst (2002a) tested this hypothesis using relative-rate tests on sequences of 21 proteins and two ribosomal RNA (rRNA) genes to examine the rates of acipenseriform molecular evolution relative to that of the teleosts. Sequences from both the nuclear and mitochondrial genomes were included. Using two different relative rate tests (the two-cluster relative-rate test of Takezaki et al., 1995 and the nonparametric relative-rate test of Tajima, 1993), sturgeon/paddlefish sequences were compared with teleost sequences relative to sequences from an outgroup (either Chondrichthyes, Dipnoi, Petromyzontiformes or Rodentia). Seventy of 81 comparisons (86%) between individual taxonomic groups showed the acipenseriform sequence evolving more slowly than the teleost sequence. The rate of change in acipenseriforms may be almost 50% slower than in teleosts (Krieger and Fuerst, 2002a). Characteristics of sturgeons and paddlefish that have been proposed previously to account for slow rates of molecular evolution in other taxa include long generation time (Kohne, 1970; Li et al., 1987; Ohta, 1993; Mooers and Harvey, 1994; Li et al., 1996) and low metabolic rate (Martin and Palumbi, 1993; Martin, 1999). The relatively slow rate of molecular evolution in acipenseriforms has implications for efforts to differentiate between species, identify stocks within species or determine evolutionary relationships among species.

4.3 Molecular Phylogeny of North American Acipenseriformes

4.3.1 Nuclear 18S rRNA Gene

The classification of the group has, historically, been based on morphological characteristics. When molecular tools were applied in early DNA based efforts to clarify the phylogeny of sturgeons, the nuclear 18S rRNA gene was one of the first genes investigated. Birstein et al. (1997) determined partial 18S rRNA gene sequences

(230 base pairs or bp) in eight species of acipenseriforms, including four North American species. They found that these sequences provided little resolution of phylogenetic relationships because of a low degree of sequence divergence among species. Later, Krieger and Fuerst (2002b) examined the phylogenetic utility of the complete 18S rRNA gene in North American sturgeons and paddlefish. After sequencing the entire gene in the ten North American species, intraindividual variation of the 18S rRNA gene was discovered in all North American sturgeons, but not in paddlefish or in other non-acipenseriform species examined (gar, bowfin, bichirs, redfish and lungfish) (Krieger and Fuerst, 2002b). Sturgeons seem to have the normal structure of the rRNA transcription unit found in other eukaryotes. Three rRNA genes are found in a single transcription unit that is tandemly repeated in the genomes of eukaryotes, so that many copies of each gene are present in an individual. Despite the presence of multiple copies, intraindividual variation of the sequence of the 18S rRNA gene is an unusual phenomenon; a process called concerted evolution homogenizes repetitive genes both within an individual and a species (Arnheim et al., 1980). Sturgeons represent the first example of 18S rDNA intraindividual variation in a vertebrate, and Krieger and Fuerst (2002b, 2004) have proposed that the presence of extra rDNA arrays in the sturgeon genome due to a polyploid ancestry allowed the formation of multiple sequence variants in sturgeons.

The intraindividual variation of a few sturgeon species has been characterized. In early attempts, the 5 half of the gene from *Acipenser oxyrinchus oxyrinchus* and *A. transmontanus* and the entire gene from *A. brevirostrum* were studied with limited success. Subsequent efforts have focused on *A. fulvescens*. Thirty DNA clones containing full-length genes isolated from *A. fulvescens* were screened by sequencing and found to contain at least 17 different sequence variants (Krieger and Fuerst, 2002b, 2004). Both individual nucleotide substitutions and insertion/deletion (indel) events were observed in the isolated sequence variants.

In phylogenetic analyses, the sturgeon sequences form a monophyletic clade compared to paddlefish or other fishes. However, these sequences were not phylogenetically useful within sturgeons, since the intraindividual variation present in sturgeons affects the utility of this gene to distinguish species or identify species relationships. The sequence variants isolated from a particular sturgeon species do not always cluster together. Instead, they intermix with variants from other sturgeon species (see Fig. 4 in Krieger and Fuerst, 2002b). At higher taxonomic levels the acipenseriform sequence variants do retain accurate phylogenetic information. The lake sturgeon sequence variant that is most similar to the *Polyodon spathula* sequence and the sequence variant most divergent from the paddlefish sequence were both used to investigate the stability of the position of the order Acipenseriformes with the Actinopterygii. When one of these two sequence variants was used with the *P. spathula* and *Scaphirhynchus platorynchus* sequences to represent the order Acipenseriformes in phylogenetic analyses, the placement of the order was consistent in relation to other fish groups, regardless of which of the two lake sturgeon sequence variants was included. The inclusion of single lake sturgeon variants alone also produced the same placement for the order, showing Acipenseriformes clustered with the Polypteriformes (Krieger and Fuerst, 2002b).

The main question related to the phenomenon of intraindividual variation is why there are so many 18S rDNA sequence variants in sturgeons when most other species examined possess only one. One possibility is that some of the sequence variants are non-functional pseudogenes, while one or a few are functional sequences. Sequence comparison, phylogenetic analyses, and relative-rate analyses were conducted with the 17 18S rRNA gene sequences isolated from *A. fulvescens* to search for evidence that some variants may be pseudogenes. In addition, RNA analysis was used to examine which sequence variants are expressed in the fish. The results from these analyses provided some evidence supporting the pseudogene hypothesis, but were not conclusive in proving any of the variants to actually be pseudogenes. The question remains open.

Phylogenetic analysis of the lake sturgeon sequence variants and the single *P. spathula* sequence indicates the presence of two major clusters of sequences. Two lake sturgeon sequence variants were grouped with the paddlefish sequence, and were named paddlefish-like alleles, while 15 sequence variants clustered together, but separate from the paddlefish sequence, and were designated non-paddlefish-like alleles (see Fig. 1 in Krieger and Fuerst, 2004). The split of the sequence variants into two groups, with one group more closely related to the functional single paddlefish sequence, suggested that paddlefish-like alleles may be functional while non-paddlefish-like alleles may be pseudogenes.

Nevertheless, sequence comparison did not provide any obvious evidence that would clearly indicate pseudogene status for any of the lake sturgeon sequence variants (Krieger and Fuerst, 2004). None of the observed sequence variants have extensive regions of non-homology or indel events when compared to the paddlefish. The number of differences among the various lake sturgeon sequence variants ranges from 6 to 72. Variable sites are distributed relatively evenly throughout the gene, with proportionally fewer variable sites located in regions of the molecule that are essential for the formation of proper secondary structure and thus functionality. Most sites showing indel events are located in regions of the molecule that form hairpin and internal loops, regions that are considered less essential for secondary structure formation and, consequently, ribosome function. In addition, 63% of the variable sites found in the lake sturgeon variants are considered either minimally conserved or non-conserved sites in a generalized eukaryotic 18S rRNA secondary structure model map of site conservation (Cannone et al., 2002). Each of these observations suggests that overall nucleotide variation among lake sturgeon 18S rRNA gene variants has occurred in the presence of natural selection, and that the variants carry changes that have little or minimal effects on a functional molecule. However, it should be noted that the remaining 37% of variable sites occur at positions that are considered to be universally conserved, highly conserved, or conserved in the secondary structure site conservation model. Further examination of nucleotide sites in regions thought to be important for the normal function of the ribosomal small subunit in the ribosome during translation (A-site, P-site, E-site and helix 27) showed almost no changes in the sequence variants and compared to eukaryotic sequences that are consistent with a standard structural diagram of eukaryotic phylogenetic conservation, indicating sequence

conservation in functional sites in the lake sturgeon variant alleles. Exceptions were observed at one position in the ribosomal A-site sequence and two positions in the P-site sequence, all three of which are considered universally conserved in eukaryotes. These latter variations were observed in the three lake sturgeon sequence variants that show the largest number of changes at universally conserved sites, making them the most probable candidates to be non-functional pseudogenes.

The results of the relative-rates test also gave some support to the pseudogene hypothesis, as did reverse transcription polymerase chain reaction (RT-PCR) analysis (Krieger and Fuerst, 2004). A relative-rate analysis was carried out to determine if the non-paddlefish-like alleles are under reduced selective constraints as compared to the paddlefish-like alleles, as would be expected if the non-paddlefish-like alleles are non-functional pseudogenes. The non-paddlefish like alleles were found to be evolving more quickly than the paddlefish-like alleles, and so appear to be under reduced selection pressure as expected of pseudogenes. Reverse transcription of lake sturgeon 18S rRNA followed by DNA sequencing of the RT-PCR product produced a single readable sequence, indicating that only one variant is expressed in the RNA of lake sturgeons in quantities large enough to be detected during sequencing. This result suggested that despite the large number of 18S rDNA alleles found in the lake sturgeon genome, there may be only one functional, expressed variant and that the other alleles may be unexpressed pseudogenes. The detected variant was identical in sequence to one of the clones isolated from the lake sturgeon genome, a paddlefish-like allele that differs by only one base from the paddlefish sequence. Although concerted evolution has failed to homogenize the numerous rDNA sequence variants found in sturgeon, expression of these variants appears to be under selective pressure.

In a more recent investigation (Krieger et al., 2006), intraindividual variation of the 18S rRNA gene has been found in all 14 Eurasian sturgeon species studied, but no variation was found in the Chinese paddlefish. The observation that intraindividual variation is present in sturgeons but absent in paddlefishes suggests that intraindividual variation arose within the Acipenseriformes after the divergence of the sturgeon and paddlefish families. Phylogenetic analysis of two polymorphic but readable segments of 18S rRNA gene sequence from 24 species of Acipenseriformes again provided no resolution of species relationships. However, patterns of 18S rDNA indel events observed within sturgeon species did correlate with proposed evolutionary relationships of certain species. For example, relevant to the North American species, *A. sturio* and *A. oxyrinchus* cluster together in molecular phylogenetic analyses (Birstein and DeSalle, 1998; Fontana et al., 2001; Ludwig et al., 2001; Birstein et al., 2002; Krieger et al., 2008). The presence of a unique indel event common to only *A. sturio*, *A. o. oxyrinchus* and *A. o. desotoi* supports the close relationship of these species. In addition, within the two readable polymorphic regions of the 18S rRNA gene that were analyzed for phylogenetic utility, there were three unique polymorphic sites shared by the three species. They also uniquely lacked variation at one other site where variation is found in all other species of *Acipenser* and *Huso*.

4.3.2 Mitochondrial DNA (mtDNA)

Although the search for nuclear markers remains an important focus for the study of sturgeon evolution, difficulties with identifying useful nuclear DNA sequences have been encountered. This appears to be due to the high ploidy level of sturgeons and the apparent slowed rates of nucleotide evolution. The lack of useful nuclear gene information means that most phylogenetic studies rely on mitochondrial DNA (mtDNA). The advantages of using mtDNA for phylogenetic studies in sturgeons and paddlefish are: (1) mtDNA is maternally inherited, essentially as a haploid molecule, and avoids potential problems caused by high ploidy levels in the nuclear genome; (2) animal mtDNA evolves more rapidly than nuclear DNA, and (3) complete mtDNA sequences are available from many fish species, including seven acipenseriform species (*P. spathula*, *Psephurus gladius*, *Scaphirhynchus albus*, *Huso huso*, *A. transmontanus*, *A. stellatus* and *A. dabryanus*), which facilitates the design of PCR and DNA sequencing primers. Many studies of acipenseriform phylogeny based on mitochondrial DNA sequences have been carried out on various subsets of species (for example: Brown et al., 1996; Birstein and DeSalle, 1998; Tagliavini et al., 1999; Krieger et al., 2000; Zhang et al., 2000; Ludwig et al., 2001; Birstein et al., 2002). However, the focus of this paper is on North American species, so the discussion will be limited to two studies that examined solely North American species. The sequence of the mitochondrial DNA control region and whole mtDNA restriction fragment length polymorphisms (RFLPs) were examined for their phylogenetic utility by Brown et al. (1996) in four species of sturgeon. They found that *A. transmontanus* and *A. medirostris* were the most closely related of the group examined, with *A. fulvescens* branching next, followed by *A. oxyrinchus*. These early results were consistent with a subsequent larger phylogenetic study by Krieger et al. (2000) that included all North American acipenseriform species and was based on four complete combined mitochondrial gene sequences (12S rRNA, cytochrome *c* oxidase subunit II, $tRNA_{Asp}$ and $tRNA_{Phe}$ genes). In the latter study, analyses using neighbor-joining, maximum parsimony, and maximum likelihood all produced similar topologies. Sister-species relationships were identified between *A. fulvescens* and *A. brevirostrum*, two eastern North American species, and between *A. transmontanus* and *A. medirostris*, two western North American species. The two *A. oxyrinchus* subspecies (*A. o. oxyrinchus* and *A. o. desotoi*), also identified as sister taxa, were very similar, separated by only three nucleotide differences in the four genes and were taxonomically located in a position basal to all other *Acipenser* species. However, on the basis of this study, it could not be determined which group held the most basal position within the North American Acipenseridae: the three species of *Scaphirhynchus* or the pair of *A. oxyrinchus* subspecies. In addition, nucleotide sequences for these four genes in all three *Scaphirhynchus* species were identical, providing no evidence for the separation of species with the genus. This tree topology agreed with that produced by a study in a larger number of sturgeon species of whole mitochondrial cytochrome *b* gene sequences (Ludwig et al., 2001), but differed slightly from the results of two studies of partial mitochondrial genes (also in larger numbers of sturgeon species) conducted by Birstein and DeSalle (1998) and

Birstein et al. (2002). In contrast to the finding that *A. fulvescens* and *A. brevirostrum* are sister species (Krieger et al., 2000), the results of Birstein and DeSalle (1998) and Birstein et al. (2002) suggested a distant relationship between lake and shortnose sturgeon. Recently, we have completed the most extensive study of sturgeon phylogeny, using data from eight mitochondrial genes (Krieger et al., 2008). In this latest study, *A. fulvescens* and *A. brevirostrum* are again identified as closely related, and the basal clade remains unresolved (Krieger et al., 2008).

4.4 Species Distinction in North America

In the United States, the amount of protection provided to a species under the Endangered Species Act is partially dependent upon its taxonomic status. Almost all North American acipenseriform species are either threatened or endangered, and there are two groups of North American sturgeons that include very closely related species or subspecies, one being *A. oxyrinchus*, with two subspecies, and the other being *Scaphirhynchus*, with three species. There has been controversy over the separate status of these taxa, prompting more focused genetic analysis of these fish.

4.4.1 A. oxyrinchus Subspecies

Gulf sturgeon (*A. o. desotoi*) and Atlantic sturgeon (*A. o. oxyrinchus*) have been considered separate subspecies, on the basis of differing habitat ranges and life history characteristics. The Atlantic sturgeon is found along the east coast of North America from the St. Lawrence river in Quebec to the St. Johns river in Florida, while the Gulf sturgeon is restricted to the Gulf of Mexico and its tributaries (Smith, 1985). Atlantic sturgeon mostly inhabit coastal marine waters, then return to natal rivers to spawn (Dovel and Berggren, 1983), while Gulf sturgeon live in their natal rivers for much of the year and only make brief wintertime excursions into the Gulf of Mexico (Wooley and Crateau, 1985). As yet, only one separating morphological character has been identified, i.e., relative spleen length (Wooley, 1985).

In the United States, the Gulf sturgeon is considered threatened, while the Atlantic sturgeon is not. Two genetic studies addressing the question of differentiation between Atlantic and Gulf sturgeons have been conducted. Ong et al. (1996) examined 203 bp of mitochondrial control region sequence, and King et al. (2001) studied seven microsatellite loci in the two taxa. Three fixed differences were detected between Gulf and Atlantic sturgeon mitochondrial control region sequences (Ong et al., 1996), while considerable divergence between the nuclear genomes of these two taxa was demonstrated with microsatellite DNA allele frequencies and diversity (King et al., 2001). The average genetic distance between the subspecies was determined to be two to three times that seen between

A. o. oxyrinchus populations (King et al., 2001). Therefore data from both mitochondrial and nuclear markers agree with the limited morphological evidence and with geographic distribution to support the subspecific status of the Atlantic and Gulf sturgeon and their separate management.

4.4.2 Scaphirhynchus Species

Shovelnose sturgeons (*S. platorynchus*) have been found throughout the Mississippi river basin and the Rio Grande river and are sympatric with the pallid sturgeon (*S. albus*) in the Missouri and Lower Mississippi rivers (to which the pallid sturgeon are restricted) (Lee, 1980a,b; Carlson et al., 1985), while the allopatric Alabama sturgeons (*S. suttkusi*) are found only in the Mobile river drainage (Burke and Ramsey, 1995). Morphological evidence supporting the presence of three separate species has been provided (Bailey and Cross, 1954; Williams and Clemmer, 1991; Mayden and Kuhadja, 1996), but their taxonomic distinction has been called into question, especially that of the sympatric shovelnose and pallid sturgeon.

Both the pallid and Alabama sturgeons are listed as endangered, and numerous studies have attempted to demonstrate genetic differentiation among the three *Scaphirhynchus* species. An allozyme study in shovelnose and pallid sturgeons by Phelps and Allendorf (1983) found the two species to be electrophoretically indistinguishable. Based on the banding patterns produced by PCR amplification with primers for three nuclear genes, Genetic Analyses, Inc. (1994) found greater differentiation between the Alabama sturgeon and the other two species than between the shovelnose and pallid sturgeon, and it was concluded that the latter two may not be distinct species. Campton et al. (2000) were able to find evidence distinguishing all three species by examining mitochondrial control region haplotypes and their frequencies, and also identified a unique substitution and haplotype that differentiated the Alabama sturgeon from the other taxa. Simons et al. (2001) conducted phylogenetic analyses of the mtDNA control region and cytochrome *b* gene sequences in all three species. Only samples of the Alabama sturgeon were recovered as a monophyletic group, and they found only low levels of sequence divergence among the species, which they interpreted to have been caused by a combination of a slow rate of molecular evolution and hybridization between shovelnose and pallid sturgeons. Based on data from six polymorphic microsatellite loci, Tranah et al. (2001) provided evidence that sympatric populations of pallid and shovelnose sturgeons at three different locations, chosen to represent the extreme of the species range, were genetically distinct from each other but that hybridization might be occurring. Recently, Tranah et al. (2004) provided additional genetic evidence from mitochondrial control region and microsatellite data that pallid and shovelnose sturgeons in the lower Mississippi river are reproductively distinct populations that are experiencing hybridization. Based on current knowledge, it appears that although Alabama and shovelnose sturgeons were initially considered conspecific (Chermock, 1955), the Alabama sturgeon is the

most genetically distinct taxon in the group, while distinguishing between shovelnose and pallid sturgeon remains problematic (at least in some areas of their range) possibly due to hybridization. Future analysis of additional markers (most likely microsatellites) may be able to provide more evidence about the levels of differentiation between pallid and shovelnose sturgeons.

4.5 Population Genetic Studies Within North American Acipenseriform Species

Population studies on North American acipenseriform species examined stock structure, levels of genetic diversity within populations, evolutionary relationships among geographic populations, gene flow and homing fidelity. Most studies have utilized one or more of the four types of markers: allozymes, whole mtDNA RFLPs, mtDNA control region sequence polymorphism, and DNA microsatellites. At present, each of the North American species has been examined in at least one population study, in spite of the difficulty of collecting specimens, although sample sizes in some studies were small. Mitochondrial DNA RFLPs and control region sequence have been the most widely used markers to date. Recently, though, much effort has been devoted to developing disomic microsatellite markers for use in North American sturgeon and paddlefish species (May et al., 1997; McQuown et al., 2000; King et al., 2001; Pyatskowit et al., 2001; Heist et al., 2002; Henderson-Arzapalo and King, 2002; McQuown et al., 2002; Welsh et al., 2003; Welsh and McClain, 2004; Welsh and May, 2006). This is because of the great potential information that microsatellite markers can provide for population comparisons. Microsatellite development has been hampered in sturgeons because of the polyploid nature of the sturgeon genome. Many potential microsatellite markers have reduced utility because they appear as more than disomic, thus complicating the interpretation of genetic inheritance and genetic variation. Nevertheless, through much effort, a battery of new disomic microsatellite markers have been developed for use in sturgeons.

As a result, microsatellite markers are being increasingly used in sturgeon population studies; papers studying all the North American species have been published, reporting results obtained utilizing these nuclear markers. Varying levels of diversity and stock structuring have been detected with molecular markers in the different species. Each species and population has unique life histories and influences and must therefore be studied and considered individually when making conservation plans. However, some generalizations about each species are beginning to emerge.

Some aspects of the population genetics of sturgeons have been reviewed by Wirgin et al. (1997) and by Robinson and Ferguson (2004) in a recent comprehensive review of population genetic studies conducted up to 2002 on North American sturgeon and paddlefish. Some earlier results are summarized below, together with an update on results that have been reported since 2002.

4.5.1 Sturgeons of Eastern North America

Molecular studies have been extensively pursued to investigate the three species of *Acipenser* that are found in eastern North America. The shortnose sturgeon (*A. brevirostrum*) and the two subspecies of the Atlantic sturgeon (*A. o. oxyrinchus* and *A. o. desotoi*) are anadromous and found in the Atlantic Ocean. The lake sturgeon (*A. fulvescens*) occurs in freshwater habitats. All three have been studied, using both mitochondrial DNA and microsatellite analysis to determine the degree of population differentiation and structure, and the degree of homing fidelity for reproductive individuals.

4.5.1.1 *A. brevirostrum*

The shortnose sturgeon (*A. brevirostrum*) is currently found in 19 estuary systems along the east coast of North America, from the St. John river in New Brunswick to the St. Johns river in Florida (Scott and Crossman, 1973; Taubert, 1980). It is a semi-anadromous fish, in that it spends more time in the rivers than other anadromous species, such as the sympatric Atlantic sturgeon. It was once fished commercially, but is now considered endangered. Each of the 19 populations is considered a distinct management unit by the US National Marine Fisheries Service, the entity that is responsible for the protection and recovery of this species (U.S. National Marine Fisheries Service, 1996, 1998). However, prior to 1995 no morphological or genetic studies had been carried out to support the designation of the 19 populations as separate units. To remedy this deficiency, a series of population genetic studies using mtDNA control region sequences have been conducted on the shortnose sturgeon (Walsh et al., 2001; Grunwald et al., 2002; Quattro et al., 2002; Waldman et al., 2002; Collins et al., 2003; Wirgin et al., 2005). These investigations studied various numbers of shortnose sturgeon populations and individuals, but came to similar conclusions. Shortnose sturgeons show moderate to high haplotypic diversity, and large numbers of mitochondrial haplotypes are observed within populations, ranging from 15 to 30. There was no evidence of genetic bottlenecking, and larger than expected effective population sizes (Quattro et al., 2002) were observed for this endangered species. Different haplotype frequencies between populations lead to the conclusion that the shortnose sturgeon has a strong stock structure along the Atlantic coast. In some cases, different haplotypes (private haplotypes) were found in different populations, so that populations from most rivers were genetically distinct. For example, Grunwald et al. (2002) found significant genetic differences among all populations sampled from 11 rivers and estuaries on the east coast of North America, and five regional groupings of populations were identified. Low gene flow and high homing fidelity (at least for females) were indicated by limited haplotype sharing among populations and a large number of private haplotypes.

All the available evidence in the case of the shortnose sturgeon suggests the presence of distinct river-specific populations and supports the conservative strategy of separate management of most shortnose sturgeon populations (Walsh et al., 2001; Grunwald et al., 2002; Quattro et al., 2002; Waldman et al., 2002; Collins et al., 2003; Wirgin et al., 2005). Waldman et al. (2002) estimated the rate of migration in *A. brevirostrum*. Levels of migration were insufficient to break down population differences between drainage systems, and were found to be intermediate between levels seen in Gulf sturgeon (lowest) and Atlantic sturgeon (highest among the trio of taxa being compared).

4.5.1.2 *A. oxyrinchus*

The two subspecies of *A. oxyrinchus* provide an interesting comparison, with respect to management issues. The Atlantic sturgeon (*A. o. oxyrinchus*) is not considered endangered (even though its populations have been reduced tremendously compared to historical levels), while the Gulf sturgeon (*A. o. desotoi*) has been listed as threatened. Both subspecies have been studied using molecular methods, primarily by mitochondrial DNA analysis.

Bowen and Avise (1990) were the first to apply molecular techniques, using mtDNA RFLP analysis, to show that Atlantic and Gulf sturgeons showed significant genetic differences from one another and should be considered genetically distinct populations. They also noted that, compared to other fishes, *A. oxyrinchus* populations showed only low levels of genetic variability. Based on the levels of genetic variation, they estimated that the effective population size of the Gulf sturgeon was of the order of 50 individuals, a very small number and a level that could potentially lead to further rapid loss of genetic variation without careful stock management.

Miracle and Campton (1995) confirmed the low levels of variability in Gulf sturgeons, using mtDNA RFLPs and mt-control region sequences, suggesting that the subspecies has undergone a genetic bottleneck. Recently, Dugo et al. (2004) used DNA microsatellites to examine stock substructuring in the Gulf sturgeon. They found significant evidence of multiple genetic stocks, even within a single river drainage system, and suggested that there may be a more widespread east-west population structure between the rivers draining into the Gulf of Mexico. Waldman et al. (2002), using data from mt-control region sequences, estimated that Gulf sturgeon populations from eight river systems exchanged genes at rates substantially below the rates seen in Atlantic sturgeons.

Population structure in the Atlantic sturgeon has been investigated in several studies (Waldman et al., 1996a,b, 2002; Wirgin et al., 2000, 2002). The earliest three studies used mitochondrial haplotype analysis, while the last two incorporated microsatellite DNA analysis to study populations. Atlantic sturgeons showed substantial haplotype diversity in most populations, although no variation was detected in the northern populations of the St. Lawrence river and St. John river in Canada. Each of

these studies came to the conclusion that Atlantic sturgeon populations were highly structured by the river drainage system, with substantial differentiation from north to south. Levels of gene flow (migration) between drainage systems were estimated to be greater, however, than those measured in equivalent populations of shortnose sturgeon from the same river systems (Waldman et al., 2002). Levels of migration were substantially greater in Atlantic sturgeons than in Gulf sturgeons.

4.5.1.3 *A. fulvescens*

The lake sturgeon (*A. fulvescens*) is one of the few species of the genus *Acipenser* that lives almost exclusively in freshwater, migrating between lakes and rivers. It has an extensive distribution, and can be found in the Great Lakes, Hudson/James Bay and Mississippi watersheds in North America. Although once abundant throughout its range, it is currently considered threatened and populations have been extirpated or severely reduced in some areas. Knowledge of the genetic diversity and stock structure of the species is invaluable to inform management decisions, especially in terms of restocking efforts in the rivers where there are still remnants of the original population.

Three early studies examined the population genetics of lake sturgeons using whole mtDNA RFLPs (Guénette et al., 1993; Ferguson et al., 1993; Ferguson and Duckworth, 1997). In contrast to the studies on the shortnose sturgeon, these studies found low genetic diversity, as only two or three haplotypes were detected, as well as little genetic divergence. There was also evidence of high gene flow, which is consistent with the life history of the species, as it migrates long distances and with the lack of population structure detected between or within drainages (Ferguson et al., 1993). The results of these studies did not support the separate management of lake sturgeon populations, although the marker system used (mtDNA RFLPs) did not possess the resolution to identify management units (Ferguson and Duckworth, 1997).

The desire for lake sturgeon conservation in the Great Lakes has prompted new study to reexamine lake sturgeon population genetics using more variable markers, especially microsatellite loci. McQuown et al. (2003) studied population differences with seven microsatellite loci. They found high levels of genetic diversity, in contrast to the earlier mtDNA analyses, and observed a general division of the population into at least three large differentiated groups, providing the first genetic evidence to support local management of lake sturgeon. However, some of the loci used by McQuown et al. (2003) were not ideal, since they showed polyploid (tetrasomic) inheritance. In a study funded by the Great Lakes Fishery Trust, Welsh and McClain (2004) standardized the use and scoring of 13 microsatellite loci for the study of the Great Lakes sturgeon. Standardization of markers was conducted to establish a common set of loci for use, and their proper analysis in this species, so that data collected during different studies by different laboratories can be combined or compared, which is a significant step toward organized cooperative conservation efforts (Welsh and May, 2006).

The 13 standard microsatellite markers were then used to conduct a population genetic analysis of lake sturgeon adults collected from 19 spawning sites throughout the Great Lakes basin (Welsh and McClain, 2004). They found a range of genetic diversity among populations in terms of the average number of alleles observed. The two rivers examined in the Hudson Bay drainage had the lowest diversity (3.61 and 3.85 average alleles) while the sample from the St. Lawrence river showed the highest diversity (5.38 average alleles). Relatively high levels of heterozygosity were maintained in all populations, again despite anthropogenic pressures. Populations from some rivers were found to be genetically indistinguishable (the Bad and White rivers, and the Detroit, St. Clair and Lower Niagara rivers), but the Bad and White rivers were also found to be genetically unique from the rest of the Great Lakes. Most spawning populations were found to be distinct from other populations, indicating that there is substantial genetic structuring and possibly spawning site fidelity in lake sturgeons. Phylogenetic analyses showed a well-supported split between the Hudson Bay and Great Lakes populations, as well as moderately supported separations within the Lake Superior basin.

The data indicate the presence of within-lake basin differences, but also possible genetic exchange among the Great Lakes sturgeons, which illustrates the importance of genetic assessment of basins and coordination among basins when considering management plans for this species (Welsh and McClain, 2004). The genetic diversity and structuring observed in this study are in contrast to the results obtained from previous studies of lake sturgeon genetic diversity based on mtDNA and illustrate the potential usefulness of hypervariable microsatellite markers.

Very recently, another study combining mitochondrial DNA analysis with microsatellites to analyze population differentiation in lake sturgeons in the upper Great Lakes basin has appeared (DeHaan et al., 2006). Eight of the standardized microsatellite loci (Welsh and May, 2006) were used to study 11 populations. DeHaan et al. (2006) report that genetic diversity in lake sturgeons, as measured by microsatellites, is high, but probably below that reported in Atlantic sturgeons. Both microsatellite and mtDNA support the existence of at least three population clusters which have a geographic component (Lake Superior, western Lake Michigan, and northern Lake Michigan – Lake Huron, the Hudson Bay, and St. Lawrence populations were not included in this analysis).

Many parts of the range of lake sturgeons have not yet been adequately surveyed, so much remains uncertain about the wide patterns of population divergence in the species. The use of a standardized set of microsatellite loci, and more sensitive mtDNA analysis, based on sequencing, rather than on RFLP techniques, should yield substantial information that can be used for species and population management.

4.5.2 Sturgeon of Western North America

Two North American species, the white sturgeon (*A. transmontanus*) and green sturgeon (*A. medirostris*), exist in river drainages connecting to the Pacific Ocean.

Compared to the eastern species, few population studies have been conducted yet on these taxa.

4.5.2.1 *A. transmontanus*

Brown et al. (1992) used mtDNA RFLP variation to characterize regional differences between white sturgeon populations in the Columbia and Fraser river drainages. While all but one of the ten RFLP genotypes were shared between populations, the frequency of different classes differed between the two drainages. In particular, the Columbia river population showed lowered levels of variability. This observation was interpreted to be associated with a possible population bottleneck due to anthropogenic exploitation, which would explain why the Columbia river population showed a lower level of variation even though sturgeon from the Columbia river are hypothesized to be the source of recolonization of the Fraser river drainage following the last glaciation. Brown et al. (1993) also examined sequence variation in the mitochondrial control region in the same two populations. Again, the Columbia river population showed lower levels of intrapopulational diversity. The populations shared most haplotypes and showed only minimal divergence. Smith et al. (2002) applied both mitochondrial sequence analysis and microsatellite analysis to study diversity more intensively in the Fraser river drainage. Their results indicate that there is significant differentiation within the Fraser river drainage; at least four subpopulations appear to exist. Clearly, much more work needs to be done on other river systems to better understand the population interrelationships of white sturgeons, and fully characterize the variation in this species.

4.5.2.2 *A. medirostris*

The green sturgeon is the least studied of the North American taxa. Brown et al. (1996) showed that green sturgeon populations contain variations for mtDNA RFLP length, with the suggestion that intraspecific levels of diversity were slightly lower than those seen in three other species (*A. transmontanus*, *A. fulvescens* and *A. brevirostrum*). Israel et al. (2004) used six microsatellite loci to study four population samples of *A. medirostris,* from the Columbia river in the north to San Pablo Bay in the south. They also applied mtDNA analysis to differentiate green sturgeon individuals from sympatric white sturgeons. Significant population heterogeneity was observed among green sturgeons, but the significant differences did not show clear geographic patterning, indicating that additional sampling will be necessary to fully understand the population-structural dynamics of the green sturgeon. It appears likely that additional breeding populations exist along the Pacific coast that may be interacting with the populations sampled in this study. It is imperative to develop additional disomic microsatellite markers for use on green sturgeons, to prevent problems in interpretation arising from the use of tetrasomic markers.

4.5.3 Genetic Studies on Other North American Acipenseriforms

Molecular analyses of other taxa have not been performed to the levels that have been applied for the three eastern species of *Acipenser*. However, studies on each of the species have provided some insight into population variation and structure.

4.5.3.1 *P. spathula*

Paddlefish (*P. spathula*) have been studied to a lesser degree than sturgeons. The earliest genetic analysis of population variability was done by Carlson et al. (1982), using allozymes. Very low levels of genetic variability were observed both within and between five populations extending from Montana to Alabama. Epifanio et al. (1996) extended the allozyme studies, added mtDNA analysis, and expanded the populations studied. They observed higher levels of heterozygosity than first reported, and a small degree of population differentiation as measured by allozymes, suggesting there are two major population clusters, fish from the Mississippi river–Pearl river and fish from the Alabama river. Mitochondrial analysis, surprisingly, showed less evidence of differentiation, with most haplotypes being shared between the regions. Szalanski et al. (2000) applied mtDNA RFLP analysis on paddlefish in the Missouri river, finding substantial variability and also lack of changes in haplotype frequencies over several years.

While microsatellite markers have been developed that can be used for paddlefish (Heist et al., 2002), we are unaware of any reported population studies using microsatellites.

4.5.3.2 *Scaphirhynchus* species

The pallid sturgeon (*S. albus*) and Alabama sturgeon (*S. suttkusi*) are considered endangered in the United States, while the shovelnose sturgeon (*S. platorynchus*) is relatively common throughout its habitat in the Missouri and lower Mississippi rivers. The first genetic analysis of *Scaphirhynchus* was reported by Phelps and Allendorf (1983), who compared *S. albus* and *S. platorynchus* using allozymes, and found the two species have low levels of genetic variation, and were electrophoretically indistinguishable. Mitochondrial DNA analysis further illustrated the problems of distinguishing these taxa, as discussed above. It has been used to examine population differentiation within *S. albus* and *S. platorynchus*. Levels of genetic variation in the mitochondrial D-loop were found to be lower than that in Atlantic or shortnose sturgeons (Campton et al., 1995). Differences between southern and northern populations of each species existed, but were at levels equivalent to the differences between the two *Scaphirhynchus* species (Tranah et al., 2001). The addition of microsatellite analysis (Tranah et al., 2004) showed greater ability to distinguish species, and gave increased weight to the possibility

of hybridization in southern populations of the Mississippi river basin. They also suggest that the population differentiation of the northern and southern populations observed using mitochondrial markers can be measured using microsatellite loci, especially in the pallid sturgeon.

4.6 Conservation Management Implications of Genetic Studies

Almost all the molecular population studies suggest some degree, often marked, of population differentiation between groups. This often occurs at the level of difference between river drainages, or between lakes in the Great Lakes basin. Whatever the ultimate cause, population differentiation has implications for potential restocking of areas in which reduced populations remain. If restocking is performed using a generic hatchery stock that is genetically different from the resident population, there is the possibility of outbreeding depression caused by hybridization between genetically differentiated groups. The continued development of new molecular techniques to more accurately measure population similarity should allow for better management decisions in the future.

4.7 Conclusion

Traditionally, allozymes, mtDNA RFLPs, mtDNA sequences and nuclear microsatellite loci have been applied to analyze the phylogeny and population genetics of species in the Acipenseriformes. The majority of studies in sturgeons and paddlefish have used mtDNA methods, partly because of difficulties in analyzing the nuclear DNA of acipenseriforms related to polyploidy. However, the challenge for the future will be the isolation of new nuclear markers in the Acipenseriformes. Some microsatellites exhibit extremely high levels of allelic variation, which is especially useful in species like sturgeons that show low overall levels of genetic variation, are recently derived, or possess geographically proximate populations for which genetic differentiation may be limited and whose significance may be difficult to quantify. As a result, much effort has gone into isolating and developing microsatellite primers with disomic inheritance patterns in sturgeon. Although often useful across species, not all markers are useful for all species and there is still a need to develop sufficient numbers of markers for those species not yet adequately covered. Microsatellite data, especially when the loci are disomic, have provided evidence for genetic differentiation and diversity in some species where mtDNA diversity was previously found to be low (as in lake sturgeons). A few researchers have been able to use both mitochondrial and nuclear DNA in population studies to increase the amount of information obtained (for example, Smith et al., 2002; Wirgin et al., 2002). New nuclear markers, including both microsatellite loci and unique protein coding loci, will also be useful for further resolution of

acipenseriform evolutionary relationships, since mtDNA has probably reached its limit of usefulness for this task and additional gene sequences from the mitochondrial genome may be unable to provide more information or clarity to unanswered questions.

In summary, studies using molecular markers are getting close to a definitive phylogeny of the sturgeons. The genomes of acipenseriforms seem to be evolving at a rate slower than that in teleosts. Information about the population substructure within species is increasing. Use of the most sensitive molecular markers shows that all species of North American sturgeons and the North American paddlefish exhibit significant evidence of population substructure that must be accounted for in management plans. Much work remains to be done to genetically define the limits of populations as management units and to understand the effects of natal watershed fidelity, anthropogenic modification of the environment, and the consequences of population augmentation by stocking on the future of these ancient but elegant fishes.

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