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[Article]

Genetic Population Structure of Remnant Lake Sturgeon Populations in the Upper Great Lakes Basin

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Abstract.—Populations of lake sturgeon *Acipenser fulvescens* have undergone dramatic declines in abundance and distribution in the Great Lakes basin and are a species of conservation concern throughout their range. While information regarding the genetic population structure of this species is critical for the development of effective management plans, little information currently exists. We examined both microsatellite and mitochondrial DNA (mtDNA) variation as a means of estimating population genetic diversity within, and the degree of spatial population structuring among, 11 remnant lake sturgeon populations in the upper Great Lakes basin. Multiple measures of genetic diversity were consistently high across populations and were not significantly correlated with estimates of current adult population size. Despite substantial population declines, life history characteristics, including longevity and iteroparity, appear to have buffered lake sturgeon populations from losses of genetic diversity. Significant levels of interpopulation variance in both microsatellite allele and mtDNA haplotype frequencies (mean genetic differentiation index = 0.055 over eight microsatellite loci; mean haplotype frequencies = 0.134 for mtDNA) were detected. Population structure is most likely a function of high levels of natal fidelity, a trend observed in other species of sturgeon *Acipenser* spp. We discuss the implications of these results with regard to the management and conservation of lake sturgeon in the Great Lakes.

Genetic studies have provided valuable information on spatial population structure for aquatic species of management and conservation concern (Beaumont 1994; Nielson 1995). In the absence of information on the degree of exchange of individuals among breeding populations, genetic data quantifying the degree of population structure can be used to infer levels of historic and contemporary gene flow (Bernatchez and Wilson 1998; Taylor et al. 2001). Levels of genetic diversity within populations can provide data related to population size (Waples 1991), variance in reproductive success (Hedgecock 1994), the effects of mating systems (Planes and Lenfant 2002; Planes et al. 2002), and anthropogenic effects, including hatchery supplementation (Ruzzante et al. 2001; Page et al. 2005).

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The degree of spatial genetic structuring can be partitioned hierarchically across macro- and microgeographic scales. For example, populations of fishes in the Great Lakes may be structured by lake basin, by river drainage, or even among tributaries within river drainages. Genetic population structure in native Great Lakes fishes has been attributed to historical factors (i.e., glacial events; Bernatchez and Wilson 1998) as well as contemporary factors related to the species' ecology (i.e., fidelity to natal spawning areas; Gatt et al. 2002; Miller 2003). Anthropogenic factors, including harvest and hatchery supplementation, have also contributed to present-day levels of genetic population structure in native Great Lakes fishes (Page et al. 2005). Conservation initiatives for numerically depressed populations inhabiting anthropogenically altered landscapes can benefit from considerations of genetic evidence for causal factors underlying apportionment of genetic variance within and among populations.

Lake sturgeon *Acipenser fulvescens* are native to three major drainages in North America: the Great Lakes, Hudson Bay, and Mississippi River (Houston

1987). Before the late 1800s, lake sturgeon were abundant throughout the Great Lakes basin (Harkness and Dymond 1961). Since the mid-1800s, overharvest, habitat loss, and construction of dams that block access to spawning grounds have led to severe declines in population abundance (Harkness and Dymond 1961; Houston 1987; Auer 1999a). Presently, lake sturgeon numbers in the Great Lakes are estimated to be less than 1% of historic levels (Hay-Chmielewski and Whelan 1997). As a result of population declines, the lake sturgeon has become a species of conservation concern throughout its native range.

In response to increased concern for lake sturgeon conservation, state, federal, and tribal agencies have developed management plans to prevent further declines and to help rehabilitate remnant populations and restore extirpated populations (Hay-Chmielewski and Whelan 1997; Wisconsin Department of Natural Resources 2000). Greater understanding of the degree of spatial structure among remnant populations will aid the development of effective management strategies for populations of lake sturgeon across the Great Lakes.

Despite the growing concern for the species' conservation status, there is limited genetic data available for lake sturgeon. Previous studies examining population structure in lake sturgeon have primarily utilized mitochondrial DNA (mtDNA) markers. These studies found levels of genetic variation to be relatively low in populations across a broad geographic range (Ferguson et al. 1993; Ferguson and Duckworth 1997) and concluded that highly variable nuclear DNA markers would be needed to detect and adequately characterize levels of genetic variation within and among populations. Recently, polymorphic microsatellite markers have been shown to be an effective means for assessing sturgeon population structure (May et al. 1997; McQuown et al. 2000, 2002; Welsh et al. 2003). Israel et al. (2004) and King et al. (2001) found significant differences in microsatellite allele frequencies among populations of green sturgeon *A. medirostris* and Atlantic sturgeon *A. oxyrinchus*, respectively. Other studies have also demonstrated the efficacy of using mtDNA markers, primarily direct sequencing, as a means of inferring population structure in shortnose *A. brevirostrum*, Atlantic, and Gulf of Mexico (Gulf) sturgeon *A. oxyrinchus desotoi* (Stabile et al. 1996; Grunwald et al. 2002; Waldman et al. 2002).

The objective of this study was to utilize recently developed, more polymorphic microsatellite and mtDNA markers to quantify the level of genetic variation both within and among remnant lake sturgeon populations in the upper Great Lakes basin. Genetic data were used to draw inferences concerning the relative importance of factors underlying present-day population structure for remnant lake sturgeon populations. This information will be useful in developing management strategies for Great Lakes populations of lake sturgeon.

Methods

Sample collection.—Adult lake sturgeon were sampled during spring spawning migrations from 1999 to 2003 in 11 Great Lakes tributaries from three different basins: the Manistee River ($n = 89$), Muskegon River ($n = 17$), Peshtigo River ($n = 54$), Fox River ($n = 46$), Oconto River ($n = 18$), lower Menominee River ($n = 47$), and Wolf River ($n = 81$) from the Lake Michigan basin; Black Lake ($n = 114$) and the St. Clair River and Lake St. Clair ($n = 50$) from the Lake Huron basin; and the Bad River ($n = 39$) and Sturgeon River ($n = 30$) from the Lake Superior basin (Figure 1). Two of the populations sampled, Black Lake (Michigan) and Wolf River (Wisconsin), are presently isolated from other Great Lakes populations because of dams that prevent immigration of new individuals from other Great Lakes populations. Lake sturgeon were captured with large dip nets, gill nets, setlines, electrofishing, and trawls. Fin clips were taken from the caudal or dorsal fin from all lake sturgeon sampled and then preserved in either tissue storage buffer (4 M urea, 0.2 M NaCl, 0.1 M tris-HCl, 5% sarcosine, 10 mM EDTA) at −70°C or dried and placed in scale envelopes and stored at ambient temperatures.

Laboratory analyses.—Total DNA was extracted from fin clips following either Puregene (Gentra Systems, Inc., Minneapolis, Minnesota) or DNeasy (QIAGEN, Inc., Valencia, California) protocols according to the manufacturer's specifications. All individuals were genotyped at eight microsatellite loci: *LS68* (May et al. 1997), *Afu68b* (McQuown et al. 2002), *Spl120* (McQuown et al. 2000), *Aox27* (King et al. 2001), *AfuG9*, *AfuG63*, *AfuG74*, and *AfuG112* (Welsh et al. 2003). Microsatellite polymerase chain reactions (PCR) were conducted in 25-μL volumes containing 100 ng of template DNA, 2.5 μL of 10× PCR buffer (1 M tris-HCl, 1 M $MgCl_2$, 1 M KCl, 10% gelatin, 10% NP-40, 10% Triton X), 0.2-mM concentration of each deoxynucleotide triphosphate (dNTP), 10 pmol of fluorescently labeled forward and an unlabeled reverse primer, and 1.0 U *Taq* polymerase. Reactions were conducted using Robocycler 96 (Stratagene, Inc., La Jolla, California) and PerkinElmer 9600 (Wellesley, Massachusetts) thermocyclers. The PCR conditions were as follows: 94°C for 2 min, followed by 30 cycles of 94°C for 1 min, 1 min for primer-specific annealing temperature, 72°C for 1 min, and a 2.5-min final extension at 72°C. Microsatellite PCR products were visualized on 6% denatured polyacrylamide gels using the Hitachi (Tokyo, Japan) FMBIO II scanner. Allele

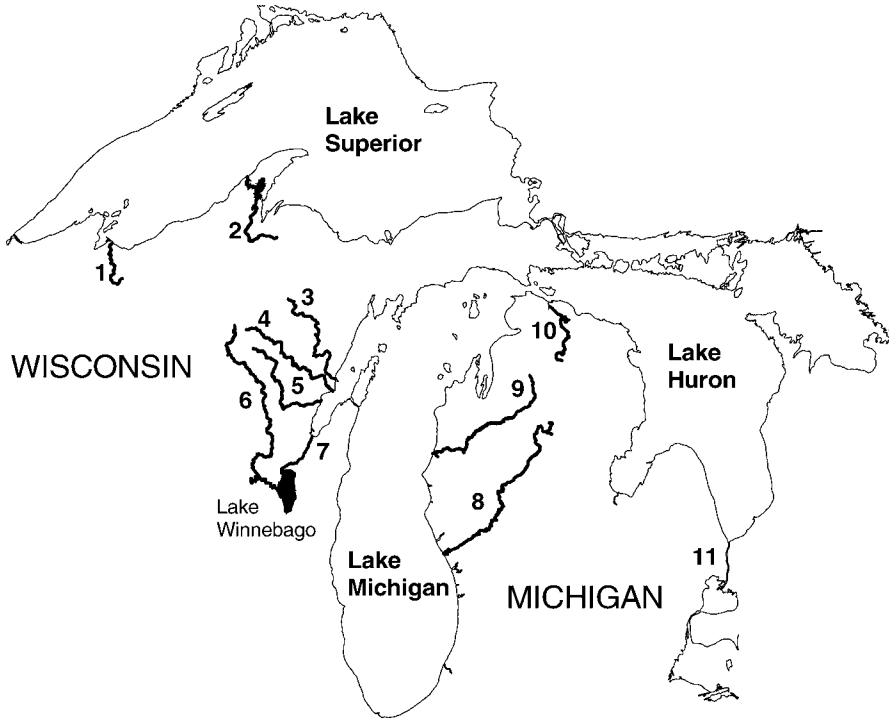

FIGURE 1.—Locations of 11 lake sturgeon spawning populations sampled in the upper Great Lakes basin, 1999–2003. Populations are numbered as follows: (1) Bad River, (2) Sturgeon River, (3) Menominee River, (4) Peshtigo River, (5) Oconto River, (6) Wolf River, (7) Fox River, (8) Muskegon River, (9) Manistee River, (10) Black Lake, and (11) Lake St. Clair and St. Clair River. Two of the populations in this study, Wolf River (6) and Black Lake (10) are isolated from other Great Lakes populations because of the construction of dams that prevent individuals from migrating into these populations.

sizes were determined using a commercially available size standard (MapMarker; BioVentures, Inc., Murfreesboro, Tennessee) and using sturgeon samples of known genotype and size. All genotypes were scored by multiple laboratory personnel.

A region of the mtDNA D-loop was also sequenced for a subset of 16–22 individuals from each population. Mitochondrial DNA analyses involved two PCRs, including an initial amplification and a subsequent sequencing reaction. The primer SturgD1F2 (5′-CAC CAT TAT CTC TAT GCG ACC-3′) was used with the primer H740 (Brown et al. 1996) to initially amplify an approximately 410-base-pair region of the D-loop downstream of a repeated region previously described by Brown et al. (1996) and Ludwig et al. (2000). Initial PCR reactions were conducted in 50-μL volumes with working concentrations of 1× buffer (67 mM tris-Cl, pH 8.0; 6.7 mM $MgCl_2$; 0.01% Tween 20), 1 μM of each primer, 1 mM dNTPs, 1.0 U *Taq* DNA polymerase, and 100 ng template DNA. Initial PCR conditions were as follows: 94°C for 2 min followed by 40 cycles of 94°C for 45 s, 50°C for 1 min, and 1 min at 72°C. This was followed by 1 min at 50°C and 5 min at 72°C. The PCR products were then run on 1% agarose gels stained with ethidium bromide to verify successful PCR amplification.

The PCR products were purified using QIAquick kits (QIAGEN), and sequencing reactions were conducted with the labeled light-strand primer (SturgD1F2) using a SequiTherm Excel II DNA sequencing kit (EPICENTRE Biotechnologies, Madison, Wisconsin) and a LI-COR IR^2 DNA sequencer (LI-COR, Inc., Lincoln, Nebraska) per the manufacturers' instructions for labeled primer sequencing. Cycle sequencing was carried out in a PerkinElmer 9600 thermocycler with the following thermal profile: 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 56°C for 30 s, and 70°C for 1 min using 100–200 fmol (quantified via spectrophotometry) of PCR product as the sequencing template. Sequences were electrophoresed through 6.5% KB+ acrylamide (LI-COR) for 7.5 h at 50 W. Sequences were scored with e-Seq v.2.0 (LI-COR) software and aligned with the program Align-IR^2 (LI-COR). Homologous haplotypes were grouped with the program Collapse v.1.1 (Chenna et al. 2003).

Unique sequences were aligned with the program Clustal W (Thompson et al. 1994).

Statistical analyses.—Characterization of levels of diversity within populations and the degree of spatial population structure was based on estimates of microsatellite allele and mtDNA haplotype frequencies. Estimates of allele frequencies and exact tests for Hardy–Weinberg equilibrium were calculated with the program GDA (version 1.0; Lewis and Zaykin 2001). The *P*-values associated with Hardy–Weinberg exact tests were adjusted for multiple comparisons with a sequential Bonferroni correction (Rice 1989). To measure genetic diversity, we also calculated allelic richness with the program CONTRIBUTE (Petit et al. 1998), which adjusts estimates of allelic diversity for differences in sample size. The total contribution that each population made to the overall gene diversity (CT) was also estimated with CONTRIBUTE. Each population's contribution to the overall diversity was partitioned into two components: the contribution of that population to the overall diversity based on its own diversity (Cs) and the contribution of a population to the overall diversity based on differentiation from other populations (Cd). The program Arlequin (version 2.0; Schneider et al. 2000) was used to calculate nucleotide diversity for mtDNA and to perform tests of selective neutrality based on mtDNA (Tajima's *D*-statistic; Tajima 1989).

Concerns have been expressed over the potential effects that low spawner abundance may have had on population levels of genetic diversity in remnant lake sturgeon populations. Using estimates of current adult population abundance (Holey et al. 2000), we used Spearman's rank order correlation coefficients to examine the relationships between estimates of population size and genetic diversity (observed heterozygosity, allelic richness, haplotype diversity). To further test the effects of low spawner abundance, we used the stepwise mutation model in the program BOTTLENECK (version 1.2.02; Cornuet and Luikart 1996) to test each population for evidence of recent population bottlenecks using the microsatellite genotypic data.

Estimates of spatial genetic population structure were derived using Weir and Cockerham's (1984) *F*-statistics calculated with the program GDA. Confidence intervals for *F*-statistics were generated based on 1,000 bootstrapping replicates over eight loci. Pairwise estimates of the genetic differentiation index (F_{ST}) and associated *P*-values were generated using the program FSTAT (version 2.9.3; Goudet 2001). The *P*-values associated with Hardy–Weinberg exact tests and pairwise F_{ST} values were adjusted for multiple comparisons using a sequential Bonferroni correction (Rice 1989). Tests for spatial variance in mtDNA haplotype frequency (Φ-statistics) were conducted with the analysis of molecular variance program (AMOVA; Excoffier et al. 1992).

PHYLIP (version 3.5; Felsenstein 1993) was used to generate Cavalli-Sforza and Edwards (1967) chord distances between all population pairs. Estimates of genetic distance were then used to generate a consensus neighbor-joining tree and the associated bootstrap values (1,000 replicates). The resulting tree was visualized with the program TreeView (Page 1996).

PHYLIP was also used to generate a tree that showed the evolutionary relationships among the 22 haplotypes observed and the associated bootstrap values for the tree (1,000 replicates). Estimates of genetic distances among haplotypes were generated using molecular information according to the most appropriate model (identified by the program MODELTEST; Posada and Crandall 1998). The distance tree was visualized with TreeView.

We used the program FLUCTUATE (version 1.4; Kuhner et al. 1995) to estimate historic population growth rates (g) and evolutionary effective female population sizes (Θ) from mtDNA sequence data. This program utilizes a coalescent approach (Beerli and Felsenstein 1999) to estimate g and the parameter Θ, which is a composite estimate of $2N_e\mu$ (or alternatively, $4N_e\mu$ for diploid data), where N_e represents the estimated evolutionary effective population size and μ represents the rate of mutation to new alleles. We randomly selected 10 individuals from each population (resulting in 11 data sets) and ran FLUCTUATE five times for each population and then calculated the mean Θ and g for each population.

To estimate migration rates between populations over evolutionary time, we used the program Migrate (version 1.6; Beerli 2002). This program also utilizes a coalescent approach to estimate evolutionary levels of migration between populations and Θ. The 11 population data sets used for the FLUCTUATE analyses were combined for the Migrate data set. We ran the program five times and report mean estimates for Θ and migration.

Results

Measures of Genetic Diversity Within Populations

Microsatellite allele frequencies and mtDNA haplotype frequencies can be found in the Appendix. The number of alleles per locus for the eight microsatellite loci in this study ranged from 3 (*Aox27* and *AfuG74*) to 12 (*Afu68b*), with a mean of 7.13. Observed heterozygosity across the 11 populations ranged from 0.541 for the Manistee River population to 0.702 for the Muskegon River population (Table 1). Allelic richness was lowest in the Menominee and Oconto populations

TABLE 1.—Measures of genetic diversity estimated from eight microsatellite loci and mtDNA sequence data for 11 lake sturgeon populations, upper Great Lakes basin, 1999–2003. Abbreviations are as follows: H_{exp} = heterozygosity expected, H_{obs} = heterozygosity observed, and A = allelic richness as in Petit et al. (1998).

Population	H_{exp}	H_{obs}	A	Number of mtDNA haplotypes	Nucleotide diversity
Lake Michigan basin					
Manistee	0.588	0.541	3.018	5	0.013
Muskegon	0.651	0.702	3.424	3	0.011
Peshtigo	0.621	0.642	3.010	8	0.027
Fox	0.600	0.588	3.117	5	0.011
Oconto	0.607	0.643	2.907	5	0.012
Menominee	0.587	0.549	2.907	9	0.021
Wolf	0.626	0.591	3.151	8	0.024
Lake Huron basin					
Black	0.678	0.607	3.358	7	0.021
St. Clair	0.690	0.664	3.332	7	0.015
Lake Superior basin					
Sturgeon	0.613	0.544	3.134	4	0.013
Bad	0.649	0.645	2.941	4	0.015

(2.907) and highest in the Muskegon River population (3.424; Table 1). Following sequential Bonferroni corrections (Rice 1989), all populations conformed to Hardy–Weinberg equilibrium at all loci, except the Manistee River and Black Lake populations. The Manistee River population was found to deviate from Hardy–Weinberg equilibrium at a single locus, *LS68*. The Black Lake population deviated from Hardy–Weinberg equilibrium at two loci, *LS68* and *Aox27*. All of these deviations were the result of a heterozygote deficiency.

A total of 22 different mtDNA haplotypes were observed (GenBank accession numbers AY947813 to AY947834). Four haplotypes (1, 2, 4, and 5) accounted for 68% of all haplotypes observed. Haplotypes 1, 2, and 8 were present in populations from each of the three major lake basins. The most common haplotype (haplotype 4) was not observed in either of the two Lake Superior basin populations. Haplotypes 3 and 14 were found exclusively in the Lake Superior populations, haplotypes 18, 19, 20, and 21 were found exclusively in the Lake Huron populations, and haplotypes 6, 7, 10, 11, 12, 13, 15, 16, 17, and 22 were found exclusively in the Lake Michigan populations. Nucleotide diversity ranged from 0.011 to 0.027 (Table 1). Tajima's D-tests for selective neutrality did not indicate any evidence of selection ($P > 0.05$).

Microsatellite DNA data showed that not all populations contributed equally to the overall gene diversity (Figure 2). The populations from Lake Michigan contributed less than average to the total diversity (indicated by negative CT values) and the populations from Lake Superior and Lake Huron contributed disproportionately to total diversity. High relative contributions from populations in the Lake Superior basin were based primarily on degree of divergence in allele frequency from other populations. In the populations from Lake Huron, high contributions to total gene diversity were based mostly on proportionally high levels of diversity within populations. Results from the program BOTTLENECK indicated that none of the populations showed evidence of a recent population bottleneck. None of the measures of genetic diversity (observed heterozygosity, allelic richness, or haplotype diversity) were significantly correlated with estimates of current population abundance (Spearman's rank correlation coefficients: 0.06, –0.04, and 0.35, respectively; $P > 0.05$).

Measures of Spatial Variation among Populations

The genetic relationships among haplotypes shown in the haplotype tree (Figure 3) were derived from the Felsenstein (1981) model of sequence evolution and revealed two groups of related haplotypes and two haplotypes that were diverged from all others (1 and 7). The two haplotype groups included haplotypes from each of the three basins (Michigan, Superior, and Huron). There was no consistent relationship between closely related haplotypes and lake basin of origin.

Overall estimates of variance in microsatellite allele frequencies were 0.102, 0.050, and 0.055 for F_{IT}, F_{IS}, and F_{ST}, respectively. All estimates were found to be statistically significant ($P < 0.05$) and had 95% confidence intervals of 0.063–0.154, 0.008–0.104, and 0.044–0.064 for F_{IT}, F_{IS}, and F_{ST}, respectively. Pairwise estimates of F_{ST} between populations ranged from 0 to 0.147, and the majority of the pairwise F_{ST} values were statistically significant (Table 2). Exceptions included the pairwise relationships between the Oconto and Peshtigo River populations, the Wolf and Fox River populations, the Wolf and Oconto River populations (all within the Green Bay region of western Lake Michigan), and the St. Clair and Muskegon River populations in Michigan.

The mean estimate of interpopulation variance in Φ_{ST} was considerably higher (0.134; $P < 0.01$) than the estimate for microsatellite loci. Pairwise values of Φ_{st} ranged from 0 to 0.378, and the majority of pairwise Φ_{ST} values were significantly different from 0 (Table 2). The exceptions were the pairwise estimates of Φ_{ST} between the Manistee River population and the Black Lake and St. Clair River populations. Several of the pairwise comparisons between the populations in Green Bay were also not statistically significant, including the Peshtigo River compared with the Fox

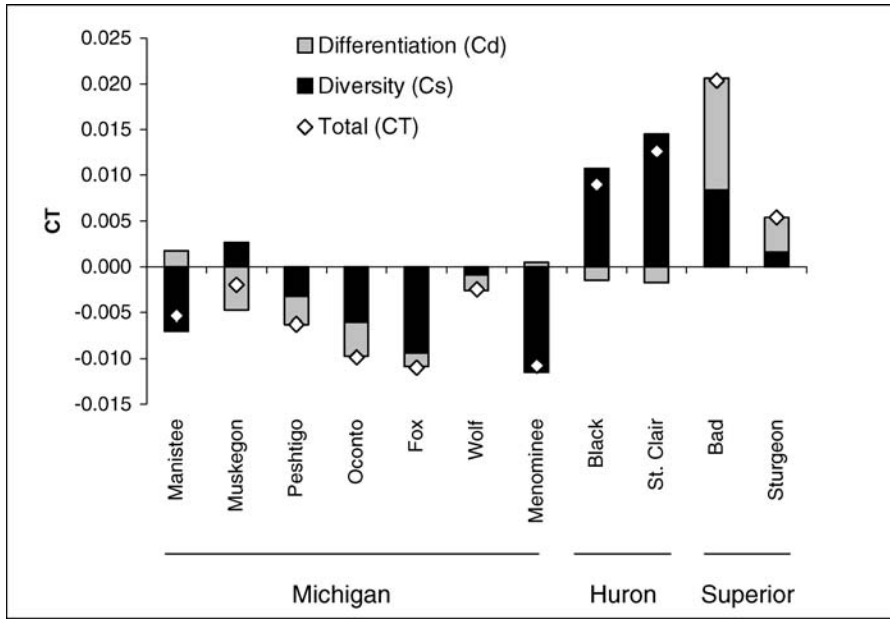

FIGURE 2.—Relative contributions of each of 11 lake sturgeon populations to total gene diversity (CT), upper Great Lakes basin, 1999–2003. Gene diversity is divided into two components: the contribution of each population to the overall diversity based on differentiation from other populations (Cd) and the contribution of each population to the overall diversity based on diversity within the population (Cs).

River populations, Menominee and Wolf River populations, the Fox River population compared with the Oconto and Wolf River populations, and the pairwise comparison between the Oconto and Wolf River populations.

The neighbor-joining tree based on Cavalli-Sforza and Edwards' (1967) chord distances revealed three population assemblages (Figure 4). One highly supported group included populations from the Lake Superior basin (Bad and Sturgeon rivers). Another highly supported group consisted of populations from the Green Bay basin in western Lake Michigan (Oconto, Peshtigo, Fox, and Wolf rivers). The third group included populations from eastern Lake Michigan and Lake Huron (Muskegon River, Manistee River, and St. Clair). The majority of the nodes on the tree showed greater than 75% bootstrap support. The relationship of the Black Lake and the Menominee River populations with the other populations is not well resolved. The Menominee River appears to have genetic affinities closer to Lake Superior than to other populations in western Lake Michigan.

Results from the FLUCTUATE program showed that the majority of Lake Michigan populations probably experienced negative growth rates (g) over geologic time. Exceptions were the Peshtigo River and Menominee River populations, which both had positive g (Table 3). The populations from Lake Huron (Black Lake and St. Clair Lake and St. Clair River) and the populations from Lake Superior (Bad and Sturgeon rivers; Table 3) also showed positive estimates of g over geologic time.

Mean estimates of Θ for the 11 populations varied by more than two orders of magnitude and ranged from 0.0004 (Oconto and Sturgeon rivers) to 0.0267 (Black Lake; Table 3). Estimates of female migration rates based on coalescent analyses of mtDNA sequences varied widely from 0 to 19,024.39 (Table 3). This probably reflects historical admixture of fish possessing evolutionarily diverged haplotypes from different Great Lakes refugia as well as contemporary gene flow. Overall, the greatest migration rates were estimated to have occurred into the Sturgeon River population, suggesting that this was an area of historical convergence as well as migration among present lake basins. The lowest migration rates were estimated for the Black Lake population, probably because of the fact that the nearby Cheboygan River was the only drainage that historically supported lake sturgeon populations in that region of Lake Huron (Holey et al. 2000).

Discussion

Gene flow greatly influences the degree of spatial variance in gene frequency among populations. Adult

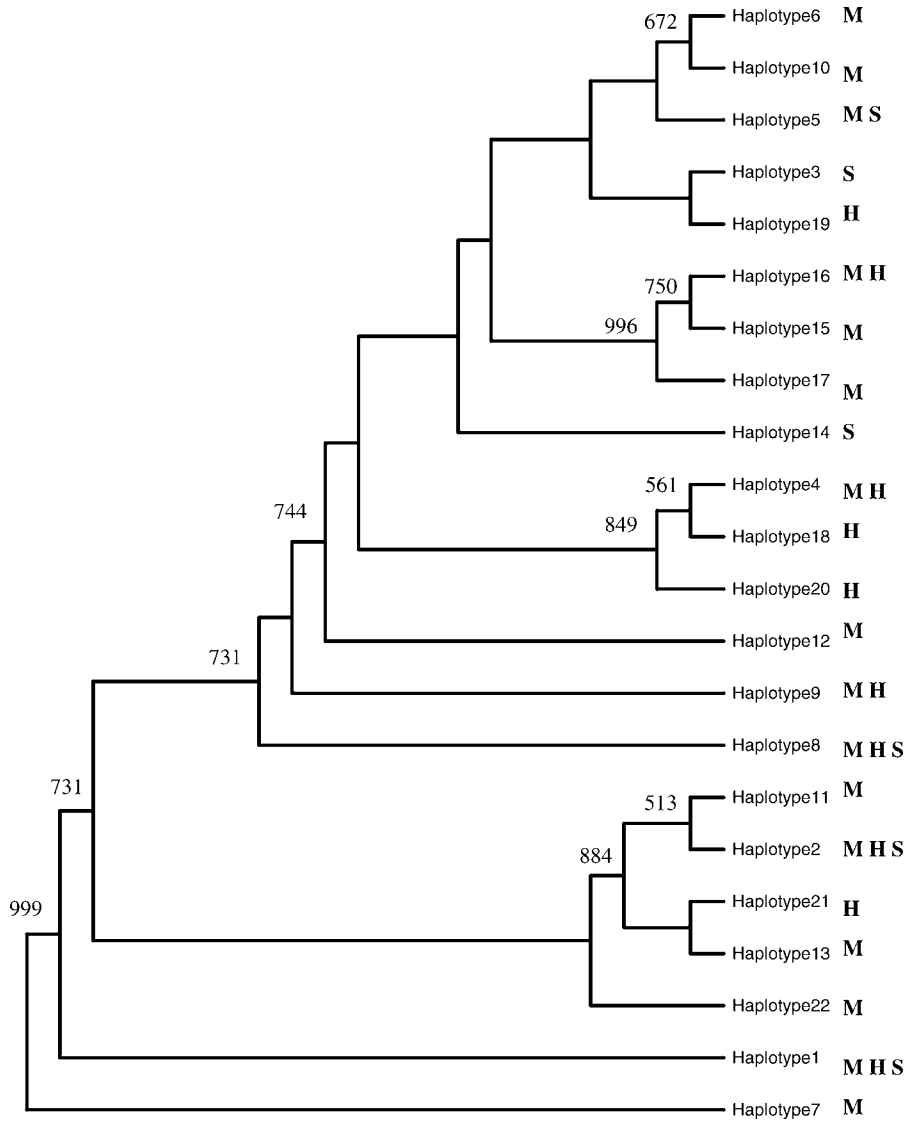

FIGURE 3.—Phylogenetic tree showing the evolutionary relationships between the 22 mtDNA haplotypes observed across 11 lake sturgeon populations, upper Great Lakes basin, 1999–2003. Bootstrap values represent the number of replicates out of 1,000 that displayed this structure. Abbreviations are as follows: M = haplotype found in the Lake Michigan basin population; H = haplotype found in the Lake Huron basin population; and S = haplotype found in the Lake Superior basin population.

lake sturgeon in the Great Lakes have been observed to move great distances (Auer 1999b), even between lake basins (i.e., Superior, Huron, Michigan) between spawning periods. Typically, species with high dispersal rates exhibit low levels of interpopulation variance in allele or haplotype frequency (Stabile et al. 1996). Alternatively, species that exhibit strong natal philopatry should show increased levels of spatial structure. Other species of sturgeon appear to exhibit some degree of natal philopatry based on tag return data (Stabile et al. 1996; Smith et al. 2002). Tag return data for lake sturgeon are limited because of infrequent spawning. However, existing data suggest that lake sturgeon do exhibit some degree of natal philopatry (Auer 1999b; Gunderman and Elliott 2004). Given the broad but fragmented distribution of lake sturgeon populations across the Great Lakes (Houston 1987; Holey et al. 2000), in the absence of genetic data lake sturgeon were believed to exhibit some level of spatial structuring.

TABLE 2.—Pairwise estimates of F_{ST} based on eight microsatellite loci (above the diagonal) and Φ_{ST} based on mtDNA sequences (below the diagonal) for 11 lake sturgeon populations, upper Great Lakes basin, 1999–2003. Asterisks indicate significant ($P \leq 0.05$) pairwise variation following Bonferroni correction.

	Population										
	Lake Michigan							Lake Huron		Lake Superior	
Population	Manistee	Muskegon	Peshtigo	Fox	Oconto	Menominee	Wolf	Black	St. Clair	Bad	Sturgeon
Manistee		0.021*	0.055*	0.069*	0.066*	0.070*	0.074*	0.047*	0.040*	0.147*	0.109*
Muskegon	0.057*		0.030*	0.025*	0.030*	0.051*	0.034*	0.025*	0.011	0.086*	0.069*
Peshtigo	0.069*	0.221*		0.024*	0.000	0.036*	0.023*	0.041*	0.040*	0.121*	0.076*
Fox	0.151*	0.273*	0.025		0.007*	0.048*	0.006	0.056*	0.038*	0.127*	0.084*
Oconto	0.180*	0.280*	0.072*	0.000		0.024*	0.007	0.046*	0.033*	0.123*	0.070*
Menominee	0.088*	0.174*	0.030	0.120*	0.140*		0.044*	0.044*	0.059*	0.115*	0.065*
Wolf	0.123*	0.243*	0.000	0.000	0.000*	0.072*		0.048*	0.037*	0.130*	0.087*
Black	0.003	0.087*	0.039	0.075*	0.095*	0.052*	0.036*		0.020*	0.080*	0.052*
St. Clair	0.020	0.222*	0.030*	0.120*	0.142*	0.113*	0.092*	0.033*		0.074*	0.056*
Bad	0.165*	0.362*	0.082*	0.211*	0.253*	0.137*	0.169*	0.146*	0.074*		0.081*
Sturgeon	0.164*	0.378*	0.088*	0.269*	0.291*	0.119*	0.225*	0.196*	0.087*	0.106*	

Anthropogenic forces that have resulted in population declines and the extirpation of populations may have concurrently influenced population levels of genetic diversity and spatial structure of Great Lakes sturgeon populations. Over the past 100 years (approximately five sturgeon generations), overharvest and habitat loss have led to population declines across the species' range (Houston 1987; Auer 1999a). Because of declines in abundance, the rate of genetic drift may have been accentuated in remnant populations. Genetic drift caused by low N_e can lead to a loss of genetic variation within populations, potentially reducing long-term viability for many remnant populations (Allendorf and Phelps 1980). Life history characteristics, including iteroparity and longevity, may have buffered lake sturgeon populations from expected losses of genetic diversity.

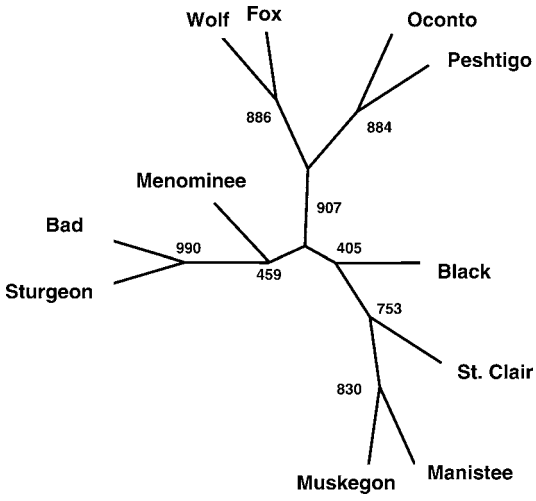

FIGURE 4.—Neighbor-joining tree based on Cavalli-Sforza and Edwards' (1967) chord distance that describes the genetic affinities among 11 lake sturgeon populations, upper Great Lakes basin, 1999–2003. Bootstrap values associated with specific nodes represent the number of replicates out of 1,000 where these groupings were evident.

Previous population genetic studies concluded that lake sturgeon have low levels of genetic diversity as well as low levels of spatial genetic structure. Ferguson and Duckworth (1997) and Ferguson et al. (1993) observed only two mtDNA haplotypes in a survey of multiple lake sturgeon populations and concluded that genetic diversity in this species was likely to be low. Contrary to these findings, we observed a total of 22 different haplotypes among the 11 populations surveyed when a more variable region of the mtDNA genome was sequenced, indicating that levels of genetic diversity are not as low as previously assumed. Additionally, estimates of genetic diversity based on nuclear microsatellite markers (heterozygosity, allelic richness) were consistently high across all populations, regardless of current population estimates. Numbers of alleles per locus and levels of heterozygosity that we observed were consistent with observations based on microsatellite data for other sturgeon species (King et al. 2001; Israel et al. 2004). These results suggest that, although populations have undergone significant reductions in numerical abundance within the past 150 years, levels of genetic diversity have not declined proportionally.

Description of Population Structure

Significant variance in microsatellite allele frequency (mean $F_{ST} = 0.055$) as well as mtDNA haplotype frequency (mean $\Phi_{ST} = 0.134$) provide evidence that remnant lake sturgeon populations in the upper Great Lakes basin are spatially genetically structured. Genetic

TABLE 3.—Estimates of lake sturgeon historical population demographic parameters and interpopulation rates of dispersal based on coalescent analyses calculated from the programs FLUCTUATE (Kuhner et al. 1995) and Migrate (Beerli 2002), upper Great Lakes basin, 1999–2003.

			Interpopulation migration rate[c]									
Population	θ[a]	g[b]	Manistee	Muskegon	Peshtigo	Fox	Oconto	Menominee	Wolf	Black	St. Clair	Sturgeon	Bad
Lake Michigan basin													
Manistee	0.0019	−26.53		4,844.29	391.15	371.01	391.15	371.01	521.53	0.00	0.00	130.38	0.00
Muskegon	0.0005	−111.20	45.42		90.84	492.62	90.84	779.17	45.42	90.84	590.47	454.21	181.68
Peshtigo	0.0016	5.01	699.57	0.00		2,098.72	699.57	4,197.44	699.57	7,103.77	1,291.11	0.00	8,394.88
Fox	0.0009	−51.28	5,550.35	1,312.02	0.00		3,061.38	1,749.36	874.68	1,312.02	874.68	437.34	1,312.02
Oconto	0.0004	−52.23	1,140.21	0.00	2,280.42	6,841.26		1,140.21	1,140.20	0.00	10,261.88	2,280.42	3,420.63
Menominee	0.0117	220.74	2,615.80	2,179.83	435.97	871.93	871.93		435.97	11,335.10	435.97	2,615.80	871.93
Wolf	0.0035	−26.53	996.79	498.40	5,482.35	3,987.17	0.00	996.79		996.79	1,993.58	0.00	0.00
Lake Huron basin													
Black	0.0267	5.61	0.00	57.08	19.03	49.03	28.54	19.03	9.51		19.03	383.44	294.89
St. Clair	0.0009	4.58	1,645.53	9,873.15	12,341.44	1,645.53	3,291.05	0.00	4,936.70	822.76		822.76	822.76
Lake Superior basin													
Sturgeon	0.0004	4.04	3,804.88	19,024.39	11,708.43	0.00	0.00	1,902.44	13,317.00	116,049.00	13,317.07		5,707.32
Bad	0.0014	32.09	2,078.01	319.69	0.00	159.85	159.85	959.08	0.00	319.69	319.69	5,274.94	

[a] $\theta = 2N_e\mu$ where N_e represents effective population size and μ represents the rate of mutation.
[b] g = population growth rate calculated from FLUCTUATE.
[c] The relative migration rate from the populations listed across the top row to the populations listed along the side.

affinities among populations revealed in the neighbor-joining tree showed high bootstrap support for three population assemblages, generally corresponding to basin of origin (Lake Superior, Wisconsin waters of western Lake Michigan, and Michigan waters of Lakes Michigan and Huron [Figure 4]). The majority of the pairwise estimates of interpopulation variance in allele frequencies (F_{ST}) and mtDNA haplotype frequencies (Φ_{ST}) were also found to be statistically significant. Data indicating significant levels of interpopulation variance in microsatellite allele and haplotype frequency suggest that spawning populations, even at microgeographic scales within lake basins, are not mixing randomly.

Several estimates of interpopulation variance, including the pairwise F_{ST} between the Oconto and Peshtigo River populations, were not significant. The neighbor-joining tree shows that these two populations are genetically similar to one another and cluster with 88% bootstrap support. The Oconto River represents one of the smallest populations in this study and it lies in close geographic proximity to the Peshtigo River. Individuals from the Oconto River may be migrating to the Peshtigo River, which supports a larger spawning population, during spawning periods where presumably greater opportunities for spawning exist.

The pairwise estimates of F_{ST} and Φ_{ST} between the lower Fox and Wolf River populations were also not significant. These populations also clustered on the neighbor-joining tree with 88% bootstrap support. While lake sturgeon were abundant historically in the lower Fox River, this population experienced similar declines as other populations during the late 1800s. In recent years, however, water quality in the Fox River has improved and lake sturgeon numbers in the lower Fox River appear to have increased; over 50 adults have been seen in spawning areas recently (Cochran 1995; Gunderman and Elliott 2004). Downstream movement of individuals from the Wolf River–Lake Winnebago system is a likely explanation for the low levels of pairwise variance (F_{ST} and Φ_{ST}) observed between these two populations (Gunderman and Elliott 2004).

While pairwise estimates of F_{ST} for the populations in Green Bay were mostly significant, they were comparatively lower than pairwise estimates of populations outside the basin. The populations in Green Bay lie in relative proximity to one another and it is likely that they exchange individuals at some low rate and, thus, are less genetically differentiated from one another than they are from populations outside the basins (i.e., Lakes Superior and Huron).

The mean estimate of Φ_{ST} (0.134) was approximately 2.5 times the overall estimate of F_{ST} (0.055). Similarly, the range of pairwise Φ_{ST} (0–0.378) we observed was greater than the range of pairwise F_{ST} values (0–0.178). In several instances, pairwise estimates of Φ_{ST} were greater than pairwise estimates of F_{ST} for comparisons between the same two populations (i.e., Oconto and Peshtigo, Black and St. Clair). These differences may be explained by the propensity for the frequencies of different genes to diverge via genetic drift. The evolutionary effective size for maternally inherited genes is fourfold less than

that for biparentally inherited genes (Avise 1994). Lower effective sizes in mitochondrial versus nuclear DNA markers will result in a more rapid accrual of variance in mtDNA relative to nuclear genes.

Although the Menominee River is a tributary of Green Bay, this population clusters with low bootstrap support with the two populations from Lake Superior. Genetic affinities between lake sturgeon from the Menominee River and populations from Lake Superior can be attributed to historical patterns of outflow from Lake Superior into the lower lake basins during glacial recession after the Wisconsin Ice Age. Lake Superior was linked to populations in Green Bay through a drainage corridor that is now the Upper Peninsula of Michigan (Underhill 1986) before ice melted in eastern Lake Superior in the area that is now the St. Mary's River. Given its location, the Menominee River would have been linked to the Lake Superior basin longer than the other Green Bay tributaries, thus allowing migration between populations from different basins.

Our findings were similar to those seen in other species of sturgeon, in which significant amounts of spatial population structure were documented. Our pairwise estimates of F_{ST} (range, 0–0.147) were similar to results seen in Pacific Coast populations of green sturgeon (pairwise F_{ST} ranged from 0 to 0.078; Israel et al. 2004). Similar to lake sturgeon, green sturgeon also disperse over great distances during nonspawning periods, yet show a high degree of fidelity to natal streams for spawning. Pairwise estimates of F_{ST} observed in Atlantic sturgeon were found to be slightly greater (range, 0.065–0.278; King et al. 2001) than our estimates for lake sturgeon. One explanation for this difference may be the greater geographic distance between Atlantic sturgeon populations compared with lake sturgeon populations in this study. Higher pairwise estimates of F_{ST} among Atlantic sturgeon populations may also be attributed to the fact that, unlike lake sturgeon, Atlantic sturgeon populations at the southern end of the species range did not undergo recent glacial events. Long-term geological stability (Bermingham and Avise 1986), coupled with strong natal philopatry, have probably contributed to high levels of genetic discordance in fish species inhabiting drainages in southern North America.

Studies have also utilized mtDNA as a means of inferring genetic population structure. Our mtDNA results indicate significant population structure ($\Phi_{ST} = 0.134$). In a study of Atlantic sturgeon, Gulf sturgeon, and shortnose sturgeon, Waldman et al. (2002) found that all three species exhibited some degree of spatial genetic population structure. While levels of variance ranged from species to species, none of the species of sturgeon surveyed appeared to exist as a single spawning population.

Factors Influencing Population Structure

Of the 11 populations surveyed, we observed a total of 22 different mtDNA haplotypes. Haplotypes were widely distributed throughout the populations surveyed and there was no consistent phylogenetic relationship between clusters of related haplotypes and population of origin either within or between lake basins (Figure 3). For example, two of the most common haplotypes (haplotypes 1 and 2), which were also evolutionarily distinct in terms of sequence divergence, occurred in all three lake basins (Figure 3). Phylogenetic relationships among haplotypes suggest that there has been extensive mixing and dispersal of lake sturgeon among Great Lakes populations after the Wisconsin Ice Age. Bernatchez and Wilson (1998) found that populations in recently deglaciated northern latitudes showed similar trends, where highly divergent haplotypes were codistributed throughout a species range and indicating secondary contact between individuals from different glacial refugia. Migration rates estimated based on coalescent analysis suggest that gene flow (or admixture of individuals from genetically diverged refugia) probably occurred over geologic time, and that contemporary gene flow among populations in close geographic proximity (i.e., among populations in western Lake Michigan) is ongoing.

The populations from Lake Superior appear to be genetically divergent from the other populations based on differences in microsatellite allele frequencies and mtDNA haplotype frequencies as well as the presence of unique mtDNA haplotypes. Results from the population tree (Figure 4) revealed that genetic distances between Lake Superior populations and populations from other lake basins were considerably greater relative to genetic distances between populations from within the same basin. Additionally, the Lake Superior populations made the greatest contribution to overall genetic diversity (CT), largely based on their degree of differentiation from the other populations. Furthermore, mtDNA haplotype 4, the most common haplotype observed, was not present in either of the two Lake Superior populations. This may be because individuals in Lake Superior originated from a separate glacial refugia than other populations in the Great Lakes. Restriction of fish into multiple refugia, followed by recolonization subsequent to the Wisconsin Ice Age, has been previously suggested for a number of native Great Lakes fish species (Underhill 1986; Mandrak and Crossman 1992).

The species' life history also plays a role in influencing contemporary levels of spatial population

structure. No barriers exist in the Great Lakes that prevent lake sturgeon from dispersing over long distances, even between lake basins. Life history characteristics, including delayed sexual maturity (approximately 15–20 years), long periods between spawning events (up to 7 years), and extensive dispersal throughout the Great Lakes between spawning periods (Houston 1987; Auer 1999b), make the collection of tag return data difficult for this species. The limited tag return data that have been recorded, however, suggest that lake sturgeon do exhibit a high degree of natal fidelity (Auer 1999b; Gunderman and Elliott 2004). This same trend has been observed in other species of North American sturgeons as well (Stabile et al. 1996; Smith et al. 2002). High levels of natal fidelity are probably one of the reasons we observe high levels of spatial population structure.

Anthropogenic forces may have played a significant role in shaping levels of genetic population structure of lake sturgeon populations in the Great Lakes. Results from the program Migrate showed that estimates of Θ ranged from 0.0004 to 0.0267. Since Θ is a composite of N_e and mutation rate ($\Theta = 2N_e\mu$), assuming an equal mutation rate among populations, populations with greater estimates of Θ have had higher N_es over evolutionary timescales. In a survey of white sturgeon *A. transmontanus*, Brown et al. (1993) estimated the mtDNA mutation rate to be 1.09×10^{-7} to 1.31×10^{-7} substitutions·nucleotide site^{-1}·year^{-1}. If the mutation rate for lake sturgeon was similar, our estimates of N_e for the populations we surveyed would range from approximately 1,800 to approximately 12,250. This suggests that N_es were relatively large, even in smaller populations. Anecdotal evidence suggests that populations of lake sturgeon were substantial before the late 1800s (Harkness and Dymond 1961). By the late 1800s, lake sturgeon had become the subject of an intensive commercial fishery and, at the same time, critical spawning and rearing habitat was being lost because of the construction of dams and pollution (Auer 1999a). By the late 1920s, levels of commercial catches had declined so significantly that nearly all commercial fisheries for this species were closed in the Great Lakes (Auer 1999a).

When populations undergo severe declines, genetic drift increases and genetic differences between populations become accentuated. Despite dramatic declines in lake sturgeon abundance, however, levels of genetic diversity, as indicated by levels of heterozygosity, haplotype and allelic diversity, and the absence of evidence for recent population bottlenecks, suggest that populations have retained genetic diversity. Levels of genetic diversity were consistent with those observed in other sturgeon species (King et al. 2001; Israel et al. 2004). These data suggest that, although population sizes have declined dramatically, genetic drift does not yet seem to be affecting genetic diversity and population structure. While this may seem surprising, it is important to consider that population sizes have only been low for 5–6 generations. If populations continue to decline or persist at low abundance, genetic drift will probably have a greater influence on population structure and levels of genetic diversity within populations.

Management and Conservation Implications

As conservation concerns increase for this species, hatchery supplementation is likely to become more widely used in attempts to reestablish extirpated populations as well as rehabilitate populations that have significantly declined in abundance. It is important to consider population structure when making decisions regarding stocking of hatchery-reared fishes. Gharrett et al. (1999) demonstrated that introgression of nonnative genetic material into locally adapted populations can lead to outbreeding depression within a matter of only a few generations. Currently, little stocking of hatchery-reared lake sturgeon has occurred within the Great Lakes. Stocking activities that have occurred in the upper Great Lakes basin have utilized source populations from both within the same lake basin and from other lake basins (e.g., Schram et al. 1999). Evidence of fundamental genetic differences among remnant populations presented in this study (e.g., Lake Superior versus Lake Michigan populations) suggest that, when possible, selection of source populations for supplementation activities should consider genetic relationships among populations. Lake basin and geographic proximity of putative source and recipient populations appear to be effective surrogate indicators of genetic relationships between populations.

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Appendix: Allele and Haplotype Frequencies

TABLE A.—Allele frequencies (based on eight nuclear microsatellite loci) and haplotype frequencies (based on mtDNA control region sequences) for 11 remnant lake sturgeon populations in the upper Great Lakes basin.

Locus and allele or haplotype	Manistee	Muskegon	Peshtigo	Fox	Oconto	Menominee	Black	Bad	St. Clair	Sturgeon	Wolf
Microsatellite loci											
LS68											
108	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.100	0.000	0.054	0.000
112	0.308	0.333	0.221	0.429	0.333	0.430	0.319	0.457	0.429	0.732	0.265
116	0.000	0.000	0.000	0.000	0.000	0.047	0.032	0.000	0.000	0.018	0.006
120	0.052	0.100	0.221	0.131	0.194	0.081	0.106	0.029	0.061	0.107	0.216
124	0.430	0.267	0.250	0.226	0.194	0.023	0.278	0.000	0.306	0.071	0.210
128	0.116	0.167	0.308	0.214	0.278	0.407	0.199	0.014	0.133	0.018	0.302
132	0.029	0.033	0.000	0.000	0.000	0.000	0.009	0.071	0.010	0.000	0.000
136	0.058	0.100	0.000	0.000	0.000	0.012	0.014	0.186	0.000	0.000	0.000
140	0.006	0.000	0.000	0.000	0.000	0.000	0.042	0.143	0.061	0.000	0.000
Afu68b											
153	0.039	0.000	0.009	0.000	0.029	0.000	0.018	0.000	0.000	0.000	0.000
157	0.000	0.029	0.046	0.060	0.147	0.205	0.005	0.100	0.000	0.286	0.040
161	0.017	0.059	0.000	0.012	0.000	0.013	0.000	0.000	0.030	0.000	0.020
165	0.017	0.059	0.028	0.024	0.000	0.051	0.009	0.000	0.020	0.036	0.033
169	0.292	0.206	0.019	0.083	0.000	0.038	0.077	0.029	0.220	0.054	0.053
173	0.337	0.235	0.157	0.083	0.059	0.244	0.355	0.243	0.190	0.232	0.100
177	0.034	0.147	0.278	0.333	0.382	0.321	0.355	0.057	0.290	0.161	0.433
181	0.011	0.000	0.194	0.179	0.235	0.026	0.023	0.029	0.000	0.071	0.140
185	0.079	0.059	0.009	0.048	0.000	0.051	0.095	0.314	0.190	0.071	0.007
189	0.107	0.176	0.083	0.167	0.088	0.026	0.059	0.000	0.020	0.000	0.140
193	0.067	0.029	0.176	0.012	0.059	0.026	0.005	0.229	0.040	0.071	0.033
197	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.018	0.000
Aox27											
130	0.860	0.853	0.833	0.944	0.889	0.964	0.737	0.686	0.650	0.732	0.864
134	0.051	0.059	0.029	0.022	0.000	0.000	0.083	0.214	0.120	0.018	0.037
138	0.090	0.088	0.137	0.033	0.111	0.036	0.180	0.100	0.230	0.250	0.099
Spl120											
254	0.635	0.500	0.402	0.438	0.529	0.477	0.279	0.214	0.470	0.214	0.500
258	0.140	0.324	0.137	0.013	0.059	0.093	0.380	0.400	0.180	0.464	0.048
262	0.062	0.029	0.196	0.050	0.176	0.198	0.135	0.186	0.060	0.089	0.089
274	0.062	0.000	0.157	0.263	0.176	0.081	0.077	0.029	0.120	0.143	0.250
278	0.000	0.000	0.010	0.013	0.000	0.081	0.067	0.157	0.020	0.018	0.008
282	0.101	0.147	0.098	0.213	0.059	0.070	0.063	0.014	0.150	0.071	0.097
286	0.000	0.000	0.000	0.013	0.000	0.000	0.000	0.000	0.000	0.000	0.008
AfuG9											
124	0.037	0.188	0.037	0.035	0.028	0.039	0.033	0.435	0.163	0.109	0.030
128	0.000	0.000	0.009	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
132	0.012	0.031	0.028	0.058	0.028	0.000	0.052	0.000	0.043	0.000	0.015
136	0.006	0.000	0.000	0.012	0.000	0.000	0.052	0.000	0.065	0.000	0.015
140	0.340	0.563	0.528	0.605	0.556	0.355	0.264	0.387	0.413	0.435	0.537
144	0.438	0.156	0.250	0.047	0.250	0.368	0.278	0.048	0.228	0.326	0.142
148	0.037	0.000	0.139	0.128	0.139	0.184	0.104	0.000	0.043	0.022	0.194
152	0.111	0.031	0.009	0.070	0.000	0.039	0.160	0.129	0.043	0.109	0.045
156	0.019	0.031	0.000	0.047	0.000	0.013	0.052	0.000	0.000	0.000	0.022
160	0.000	0.000	0.000	0.000	0.000	0.000	0.005	0.000	0.000	0.000	0.000
AfuG63											
127	0.523	0.375	0.352	0.278	0.250	0.293	0.406	0.100	0.280	0.185	0.224
135	0.029	0.156	0.046	0.144	0.111	0.037	0.000	0.000	0.100	0.000	0.103
139	0.326	0.219	0.315	0.400	0.278	0.402	0.335	0.343	0.300	0.537	0.506
143	0.123	0.188	0.287	0.178	0.361	0.268	0.259	0.543	0.300	0.222	0.167
147	0.000	0.063	0.000	0.000	0.000	0.000	0.000	0.014	0.020	0.056	0.000
AfuG74											
218	0.770	0.719	0.849	0.793	0.806	0.771	0.626	0.638	0.594	0.667	0.664
222	0.029	0.063	0.038	0.000	0.028	0.214	0.090	0.207	0.042	0.185	0.041
226	0.201	0.219	0.113	0.207	0.167	0.014	0.284	0.155	0.365	0.148	0.295
AfuG112											
240	0.000	0.000	0.000	0.012	0.000	0.000	0.000	0.000	0.000	0.000	0.000
244	0.372	0.294	0.230	0.256	0.222	0.419	0.350	0.286	0.303	0.200	0.215
248	0.000	0.000	0.000	0.012	0.000	0.041	0.005	0.000	0.000	0.040	0.013
252	0.116	0.265	0.060	0.198	0.111	0.135	0.168	0.167	0.105	0.080	0.241
256	0.000	0.000	0.010	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
260	0.207	0.206	0.400	0.267	0.417	0.324	0.248	0.071	0.263	0.540	0.272
264	0.061	0.029	0.070	0.105	0.056	0.068	0.154	0.476	0.145	0.140	0.114
268	0.232	0.206	0.230	0.151	0.194	0.014	0.075	0.000	0.184	0.000	0.146

TABLE A.—Continued.

Locus and allele or haplotype	Manistee	Muskegon	Peshtigo	Fox	Oconto	Menominee	Black	Bad	St. Clair	Sturgeon	Wolf
mtDNA haplotypes											
1	0.421	0.667	0.063	0.063	0.000	0.200	0.278	0.000	0.150	0.095	0.000
2	0.263	0.000	0.188	0.188	0.000	0.050	0.111	0.368	0.400	0.524	0.000
3	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.158	0.000	0.048	0.000
4	0.211	0.278	0.125	0.125	0.500	0.050	0.278	0.000	0.250	0.000	0.308
5	0.000	0.056	0.125	0.125	0.136	0.300	0.000	0.053	0.000	0.333	0.077
6	0.000	0.000	0.125	0.125	0.136	0.000	0.000	0.000	0.000	0.000	0.154
7	0.000	0.000	0.125	0.125	0.136	0.000	0.000	0.000	0.000	0.000	0.154
8	0.000	0.000	0.000	0.000	0.000	0.050	0.056	0.316	0.050	0.000	0.000
9	0.000	0.000	0.000	0.000	0.000	0.100	0.167	0.000	0.000	0.000	0.077
10	0.053	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.077
11	0.053	0.000	0.000	0.000	0.091	0.100	0.000	0.000	0.000	0.000	0.000
12	0.000	0.000	0.125	0.125	0.000	0.000	0.000	0.000	0.000	0.000	0.000
13	0.000	0.000	0.000	0.000	0.000	0.100	0.000	0.000	0.000	0.000	0.000
14	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.105	0.000	0.000	0.000
15	0.000	0.000	0.063	0.063	0.000	0.050	0.000	0.000	0.000	0.000	0.000
16	0.000	0.000	0.063	0.063	0.000	0.000	0.056	0.000	0.000	0.000	0.000
17	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.077
18	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.050	0.000	0.000
19	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.050	0.000	0.000
20	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.050	0.000	0.000
21	0.000	0.000	0.000	0.000	0.000	0.000	0.056	0.000	0.000	0.000	0.000
22	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.077