Determination of occurrence of hybridization of San Juan River razorback sucker through genetic screening of larval fishes

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Executive Summary – Hybridization is a well-documented threat to the persistence of native suckers throughout the southwestern United States. The objective of this study was to develop DNA-based genetic markers to assess hybrid status of wild-caught razorback sucker (*Xyrauchen texanus*) larvae in the San Juan River of northwestern New Mexico, southern Colorado and Utah, USA. Wild-caught sucker larvae were screened via polymerase chain reaction (PCR) for genetic variation at two microsatellite DNA loci encoded in the nuclear genome, and a mitochondrial (mt) DNA marker encoded in the control region of the mitochondrion. Nuclear markers are biparentally inherited and mtDNA is inherited exclusively through the matriline, and thus, it is possible to detect the presence of hybrids and the female parent of a hybrid individual when both marker classes are used in concert. One microsatellite (RBS-2H2) exhibited fixed genetic variants (alleles) among larval fishes surveyed, and was diagnostic for species and *F1* (1st filial generation) hybrids. The second microsatellite locus (RBS-5H2) exhibited broadly overlapping allelic variants among species and was less useful for diagnosing hybrids, but is potentially useful for studying intraspecific population genetics. Fixed differences among species were identified in the mtDNA control region, initially by nucleotide sequencing, and then assaying PCR products with restriction endonucleases. Classification of individual larvae was limited to putatively genetically pure individuals and putative *F1* hybrids. Of 61 larval fish assayed, 12 larvae were identified as genetically pure flannelmouth sucker (*Catostomous latipinnis*), 39 larvae were genetically pure bluehead sucker (*Pantosteus discobolus*), eight larvae were genetically pure razorback sucker (*Xyrauchen texanus*), and two larvae were identified as bluehead sucker × flannelmouth sucker *F1* hybrids. The female parents of hybrid individuals were XXXX. No razorback sucker *F1* hybrids were identified by genetic screening. One adult, identified morphologically and genetically as flannelmouth sucker, possessed a bluehead sucker mtDNA D-loop haplotype (i.e., allele), consistent with past introgression of flannelmouth and bluehead suckers in the San Juan River.

Introduction

Background

Hybridization between species of fish is a major conservation concern in the freshwaters of North America (Perry et al. 2002). In a review of the conservation status of North American freshwater fishes, Miller et al. (1996) indicated that 15 extinctions could be attributed, in part, to hybridization. Introgressive loss of native genomes can occur when closely related but historically allopatric species are brought into secondary contact by human-caused introductions (Rhymer and Simberloff 1996). However, habitat disturbance can facilitate hybridization between native, sympatric species that have historically maintained reproductive isolation. This is particularly true when habitat disturbance limits spawning habitat, so that species that may have segregated spatially in the past are now forced to spawn in proximity (Hubbs 1955).

This study focuses on hybridization between three native, historically sympatric catostomid species. Razorback sucker (*Xyrauchen texanus*), flannelmouth sucker (*Catostomous latipinnis*), and bluehead sucker (*Pantosteus discobolus*) are native to the San Juan River basin. Hybrids between razorback and flannelmouth suckers were first identified by Hubbs and Miller (1953). The earliest of these specimens was collected in 1889 by Jordan and Evermann (although originally identified as *Xyrauchen uncompahgre*). Since then, putative *X. texanus* x *C. latipinnis* have been reported from many areas within the Colorado River basin (Douglas and Marsh 1998, and references therein). Identification of hybrids was based on intermediate morphological characters, particularly lateral line scale count and a small nuchal keel. Morphological characters may be ambiguous when it comes to identification of hybrids, however. Ancestral polymorphism may give the impression of intermediacy when hybridization has not occurred (Smith 1992). Furthermore, the roles of common environment and phenotypic plasticity are not well understood for most complex morphological traits. Therefore, genetic characters such as allozymes, mitochondrial DNA, and nuclear DNA markers are often more reliable characters for diagnosing interspecific hybrids (Campton 1987).

Introgression between *C. latipinnis* and *X. texanus* has been demonstrated with protein and mitochondrial DNA markers. Buth et al. (1987) reported intermediacy

between *X. texanus* and *C. latipinnis* at two of 21 allozyme loci examined. They were not able to conclusively distinguish introgression from ancestral polymorphism as the cause for the observed distribution of alleles, but their data are consistent with past hybridization between the two species. Based on restriction fragment length polymorphism (RFLP) data, Dowling et al. (1996) found *C. latipinnis* mitochondrial DNA in individuals that were otherwise identified as *X. texanus*, a result that is consistent with past introgression between the two species. They were able to compare RFLP data from other members of *Catostomus*, which allowed them to rule out ancestral polymorphism as a cause these observations.

In this study, genetic determination of hybrid status was conducted on larval fishes. Genetic screening of fish larvae with allozymes is difficult because the approach requires relatively large amounts (100 – 500 mg) of biologically active protein for genetic screening. Whole larvae are often much smaller than the tissue samples required for allozymes. This problem was circumvented by focusing on nuclear of mtDNA-based markers that can be amplified from very small tissue samples using the polymerase chain reaction (PCR).

Objectives

To achieve recovery of razorback sucker, it is essential to develop baseline genetic information that will document whether hybridization among re-established razorback sucker, flannelmouth sucker, and bluehead sucker is occurring. This objective was identified in the San Juan River Basin Recovery Implementation Program Plan Item (5.3.7 and 5.3.7.1). These objectives are designed to characterize the genetic makeup of endangered fish species with the goal of maintaining the genetic diversity of these taxa. The objective of our 2002 study was to determine the extent to which hybridization has occurred in the San Juan River between *X. texanus, C. latipinnis*, and *P. discobolus* by genetically screening larval fishes collected in the San Juan River in FY 2002. Three genetic markers were developed for identifying hybrids. Two nuclear microsatellite markers and one mitochondrial marker were used to identify the occurrence and frequency of *F1* hybrids, and to identify the female parent of individual hybrid offspring, respectively.

Study Area and Specimens examined

All larval fishes used in this genetic study were made as part of the MSB San Juan River larval razorback sucker survey. The sampling area was the San Juan River between River Mile 142 (former site of the Cudei Diversion Dam) and the Clay Hills boat landing (ca. RM 2.8) just above Lake Powell in Utah. As in all post 1999 larval fish sampling efforts undertaken by the MSB, the study included making collections in reaches of the San Juan River under the jurisdiction of the National Park Service. A total of 61 larval specimens were screened for genetic variation at three loci (Appendix I).

In addition to larval fishes, genetic variation was examined at three marker loci using DNA samples obtained from adult fishes. The objective of adult screening was to optimize rapid screening methods for marker loci (prior to examining larval fishes) and examine variability patterns of genetic loci in individuals that were positively identified based on morphological characters. Fin clips were taken either from wild-caught flannelmouth (n = 28) and bluehead (n = 20) suckers (collected by David L. Propst, New Mexico Department of Game and Fish – see Appendix II for details), or from hatchery-spawned razorback suckers (n= 10) (provided by Heather L. Parmeter, MSB Division of Fishes—Appendix II).

Methods

The general strategy for genetic screening of larval and adult individuals was to first isolate DNA from each individual (either whole larvae or a fin clip from adults), and then to screen individual DNA samples for microsatellite DNA variation using PCR. Two microsatellite loci, RBS 5H2 and RBS 2H2 (Turner et al. unpublished) were screened simultaneously using multiplex PCR and fluorescein-labeled PCR primers (see Appendix III for additional details of molecular methods). The length in number of base pairs of each PCR product (i.e., allele) was determined using an ABI 377 automated sequencer and appropriate size standards. Examination of variation in allele lengths of nuclear DNA microsatellites permitted identification of putatively 'pure' individuals, and *F1* hybrids. This is because allelic lengths exhibited fixed differences among species at locus RBS 2H2.

Following microsatellite characterization, PCR-based techniques were employed to amplify a portion of the mtDNA D-loop (about 410 bp) for nucleotide sequencing. Seven adult razorback suckers, one larval bluehead sucker, and one larval flannelmouth sucker were sequenced and mapped to identify presence/absence of species-specific sites that are cut with restriction endonucleases. This analysis identified sites that could be differentially cut (depending on the species) with restriction enzymes EcoRI and AvaII, yielding species-specific fragment patterns (Figure 3). To verify that restriction sites were species-specific, we screened a subset of genetically pure individuals from each species (n = 14 of 61 larval samples). The *F1* hybrid individuals (n = 2) were then screened to determine the female parent of each hybrid offspring (Dowling et al. 1996). A subset of adult fishes were also screened for variation at the mtDNA D-loop (n = 4; Appendix II)

Results and Discussion

Microsatellite loci developed in this study provided consistent and reliable characterization of genotypes of larval (Appendix I) and adult (Appendix II) suckers, but differed markedly in patterns of variation across loci. Locus RBS 5H2 was more variable than locus RBS 2H2 compared across wild-caught larvae (Fig. 2) and adults and larvae pooled (Fig. 3). Twelve unique alleles were identified at RBS 5H2 in larval fishes compared to 7 identified in RBS 2H2, and 18 alleles were identified in pooled samples compared to 12 in RBS 2H2. No fixed differences were identified among species at locus RBS 5H2. Considering only larval fish, Nei's (1987) measure of genetic diversity was similar in bluehead sucker (h = 0.875) and flannelmouth sucker (h = 0.853), but razorback suckers (h = 0.56) were substantially less diverse at locus RBS 5H2. Nei's measure of genetic diversity is a composite measure that accounts for both the number of distinct alleles and the spread of frequencies across allelic classes. Genetic diversity measured in this way ranges from values of h = 0 (a single allele is fixed in all individuals) to h = 1 (all alleles are represented in equal frequency in the population). This value is expected to be roughly proportional to the genetic effective population size, provided that rates of mutation are similar among species (Nei 1987, Slatkin 1995), and thus differences in h probably reflect differences in effective size between endangered

razorback suckers versus more abundant bluehead and flannelmouth suckers. Analysis of genetic variance (*F*-statistics) among species indicated statistically significant frequency differences at locus RBS 5H2 when razorback sucker was compared to the other San Juan sucker species, indicating the potential of this locus to contribute to species identification using sophisticated 'assignment test' type approaches if used in concert with additional marker loci (e.g., Banks and Eichert, 2000).

Locus RBS 2H2 was substantially less diverse than locus RBS 5H2 for all species, but exhibited fixed allelic differences among species when larval samples were considered (Fig. 2). Larval razorbacks in the San Juan were fixed for the '320' allele at locus RBS 2H2 (Appendix I), and exhibited Nei's diversity $h = 0.00$. Genetic diversity was higher in flannelmouth ($h = 0.33$) and bluehead suckers ($h = 0.05$). Analysis of locus RBS 2H2 in adult samples revealed that alleles 316 and 318 were shared between flannelmouth and razorback suckers, but were present in very low frequencies in both species (Fig. 2). This raises the question of whether shared alleles among species arose by convergent evolution or through introgressive hybridization. This question was evaluated by determining female parentage using the mtDNA d-loop assay for individuals that exhibited shared alleles, and is discussed below.

Fixed differences at this locus provided unambiguous assignment of parentage for larval fishes and putative *F1* hybrids. Of 61 larval fish assayed, 12 larvae were identified as genetically pure flannelmouth sucker, 39 larvae were genetically pure bluehead sucker, eight larvae were genetically pure razorback sucker, and two larvae were identified as bluehead sucker × flannelmouth sucker *F1* hybrids. In general, larval razorback suckers exhibited lowered genetic diversity compared to adults in terms of the number of alleles. At locus RBS 5H2, four unique alleles were identified in eight larval razorback sucker samples, and 7 unique alleles were identified from individuals spawned at the Willow Beach National Fish Hatchery (n = 5) and Dexter National Fish Hatchery and Technology Center (n = 5), respectively. Differences among larval and hatchery-reared samples were more marked for locus RBS 5H2. A single allele '320' was identified from larval samples, but 6 distinct alleles were identified in hatchery-reared stocks. This difference in genetic diversity may suggest that only a few razorbacks are spawning in the wild, or that there is differential reproductive success among breeders

(Turner et al. 2002). Much larger samples sizes collected over multiple years will be required to definitively address this issue, however.

Analysis of the mtDNA D-loop region indicated that fourteen larvae identified as genetically pure by microsatellite analysis, also harbored the appropriate mtDNA D-loop restriction site haplotype. The female parent of *F1* hybrid larvae, identified by microsatellite analysis, was XXXXX. For adults, mtDNA variation was evaluated by nucleotide sequencing for seven of 10 hatchery-spawned razorback suckers. Six unique haplotypes were identified out of seven fish surveyed, but all lacked Eco RI and Ava II restrictions sites found on flannelmouth sucker and bluehead sucker mtDNA D-loop fragments. Four adult flannelmouth suckers that possessed alleles shared with razorback suckers at locus RBS 2H2 were found to have mtDNA d-loop patterns consistent with flannelmouth sucker mtDNA in three of four cases. One individual identified morphologically and genetically (based on microsatellites) as flannelmouth possessed a bluehead mtDNA D-loop fragment. This is consistent with an introgression event that occurred prior to the present generation (i.e., the individual in question is not an *F1* hybrid – see Dowling et al. 1997 for additional discussion).

Summary and Conclusions

This study successfully developed three genetic markers suitable for screening genetic variation in suckers of the San Juan River. One nuclear microsatellite marker and the mtDNA D-loop fragment exhibited fixed or nearly fixed differences among species. Of eight larval razorback suckers sampled, none exhibit patterns of genetic variation consistent with hybrid parentage. Only two *F1* hybrid individuals were identified out of 61 larval fish assayed, and both were flannelmouth × bluehead F1 hybrid individuals. The female parent of these individuals was XXXXX. One adult fish, identified genetically and morphologically as flannelmouth sucker (DLP 4961—see Appendix II) exhibited a bluehead sucker mtDNA D-loop fragment. Taken together, these results confirm the presence of *F1* and introgressive (i.e., backcrossed) hybrids among bluehead and flannelmouth suckers in the San Juan River. However, within the limits of detection of our genetic system, there is no indication that any hybrids exist among razorback larvae captured in the San Juan River or in hatchery-spawned larvae/adults sampled.

Literature cited

Banks, M. A., and W. Eichert. 2000. WHICHRUN (version 3.2): A computer program for population assignment of individuals based on multilocus genotype data. Journal of Heredity 91: 87-89.

Buth, D. G., R. W. Murphy, and L. Ulmer. 1987. Population differentiation and introgressive hybridization of the flannelmouth sucker and of hatchery and native stocks of the razorback sucker. Trans. Am. Fish. Soc. 116:103-110.

Campton, D. E. 1987. Natural hybridization and introgression in fishes: methods of detection and genetic interpretation. *In* N. Ryman and F. Utter (eds.) Population genetics and fishery management. University of Washington Press, Seattle. P. 161-192.

Douglas, M. E. and P. C. Marsh. 1998. Population and survival estimates of *Catostomus latipinnis* in northern Grand Canyon, with distribution and abundance of hybrids with *Xyrauchen texanus*. Copeia 1998:915-925.

Dowling, T. E., W. L. Minckley, and P. C. Marsh. 1996. Mitochondrial DNA diversity within and among populations of razorback sucker (*Xyrauchen texanus*) as determined by restriction endonuclease analysis. Copeia 1996:542-550.

Hubbs, C. L. 1955. Hybridization between fish species in nature. Syst. Zool. 4:1-20.

Hubbs, C. L. and R. R. Miller. 1953. Hybridization in nature between the fish genera *Catostomus* and *Xyrauchen*. Pap. Mich. Acad. Sci. Arts Lett. 38:207-233.

Miller, R. R., J. D. Williams, and J. E. Williams. 1989. Extinctions of North American fishes during the past century. Fisheries 14:22-38.

Nei, M. 1987. Molecular evolutionary genetics. Columbia University Press, New York.

Perry, W. L., D. M. Lodge, and J. L. Feder. 2002. Importance of hybridization between indigenous and nonindigenous freshwater species: an overlooked threat to North American biodiversity. Syst. Biol. 51:255-275.

Rhymer, J. M. and D. Simberloff. 1996. Extinction by hybridization and introgression. Annu. Rev. Ecol. Syst. 27:83-109.

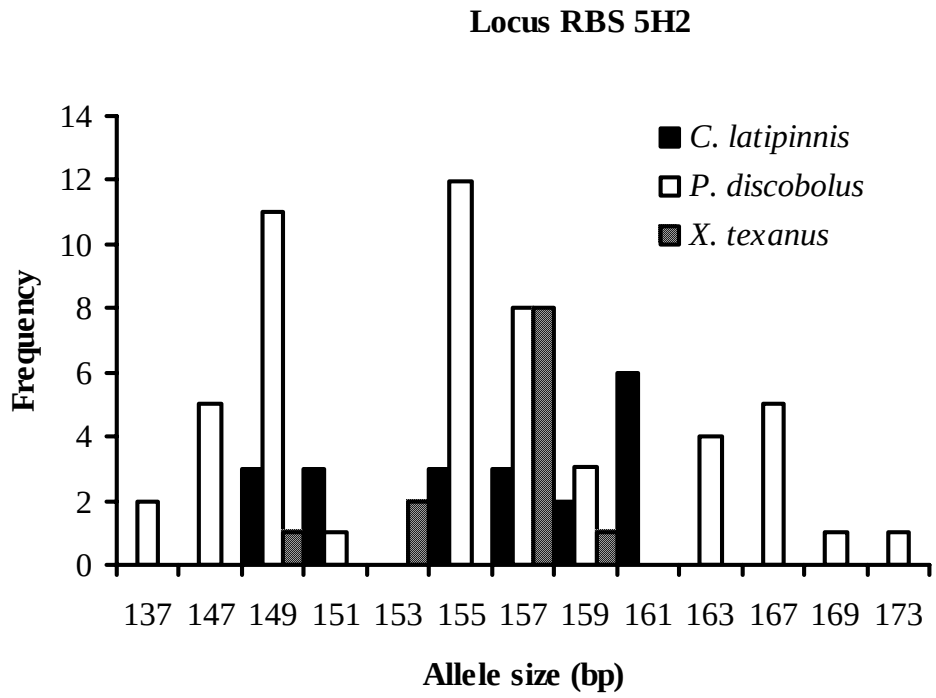
Slatkin, M. 1995. A measure of population subdivision based on microsatellite allele frequencies. Genetics 139: 457-462.

Smith, G. R. 1992. Introgression in fishes: significance for paleontology, cladistics, and evolutionary rates. Syst. Biol. 41:41-57.

Turner, T. F., T. E. Dowling, R. E. Broughton, and J. R. Gold. Microsatellite loci from the endangered razorback sucker (*Xyrauchen texanus*) are widely applicable to conservation genetics of catostomid fishes. Unpublished manuscript.

Turner, T. F., J. P. Wares, and J. R. Gold. 2002. Genetic effective size is three orders of magnitude smaller than adult census size in an abundant, estuarine-dependent marine fish (*Sciaenops ocellatus*) Genetics 162: 1329-1339.

Figure 1. Microsatellite allele frequency histograms by locus for larval fish samples collected in the San Juan River during FY 2002. Note broad overlap of alleles among species at locus RBS 5H2, and fixed differences among species at locus RBS2H2.



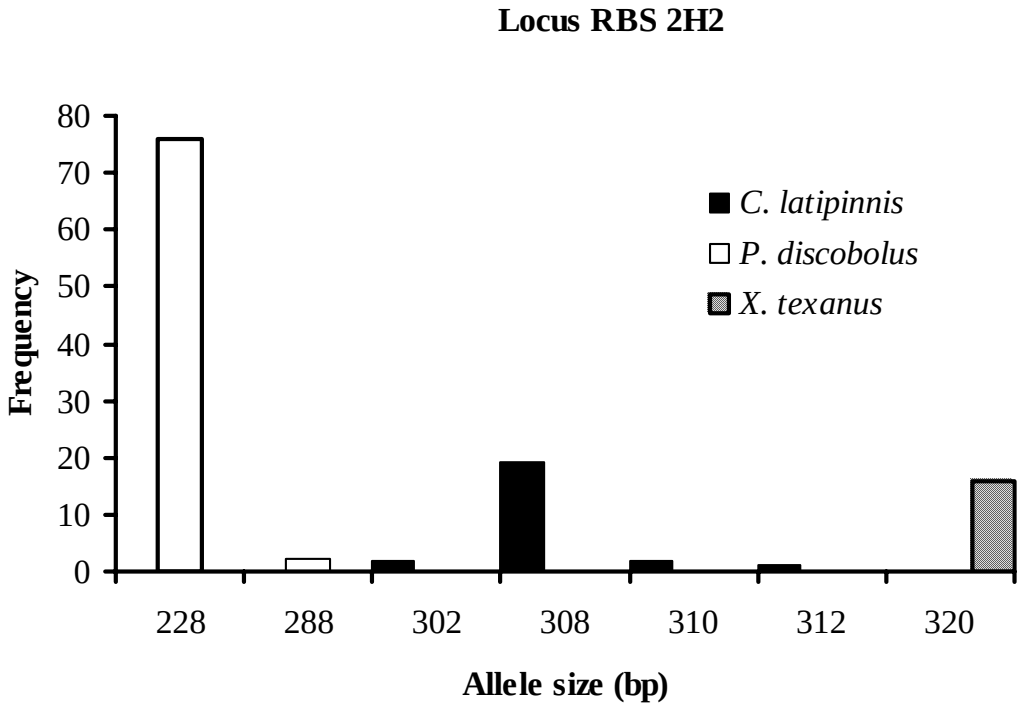
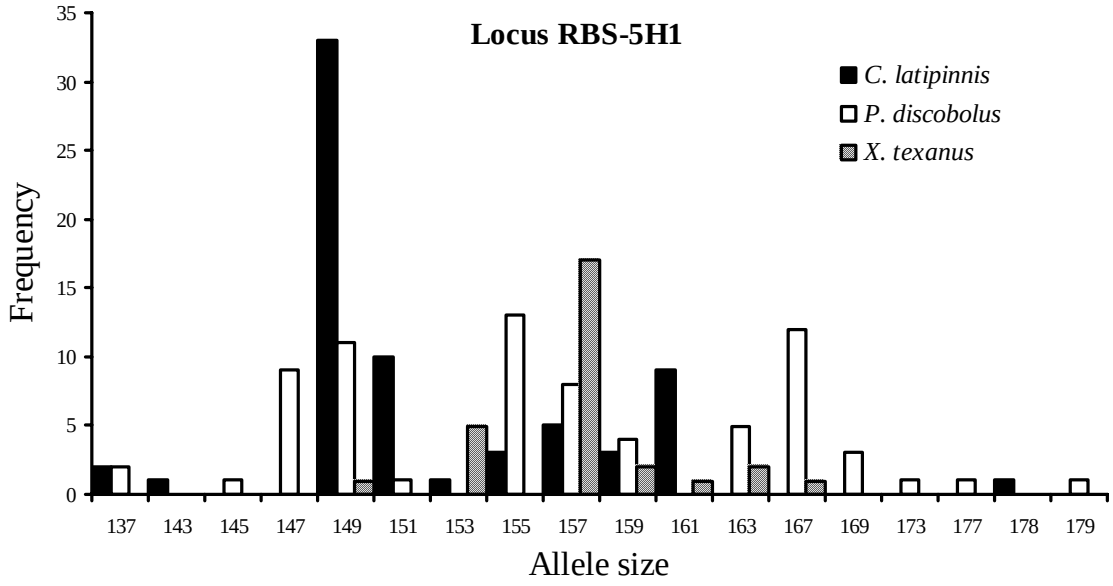


Figure 2. Microsatellite allele frequency histograms by locus for all samples (larval fish and adults) pooled.



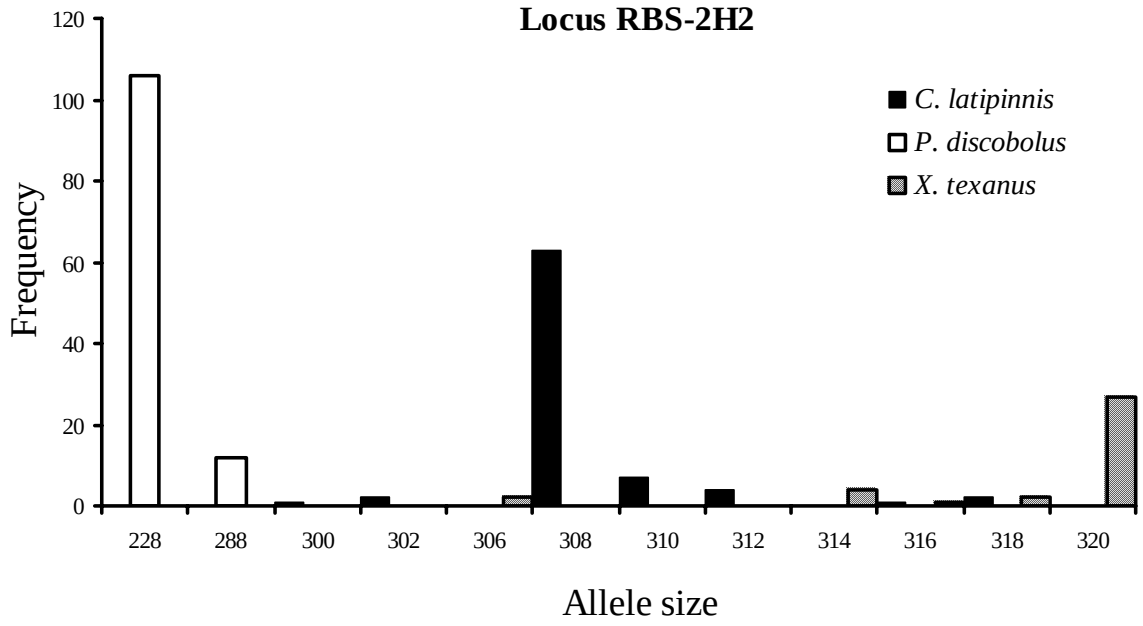
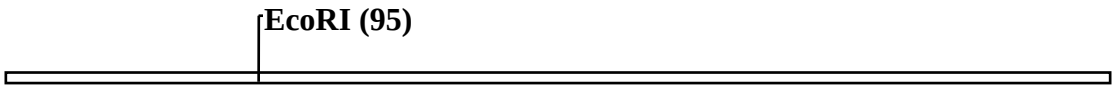


Figure 3. A map of restriction fragments generated by cutting mtDNA d-loop PCR products with two restriction enzymes, EcoRI and AvaII. The total fragment length is 410 base pairs. EcoRI cuts d-loop fragments isolated from flannelmouth and bluehead sucker, but not razorback sucker. AvaII cuts only bluehead sucker mtDNA d-loop.

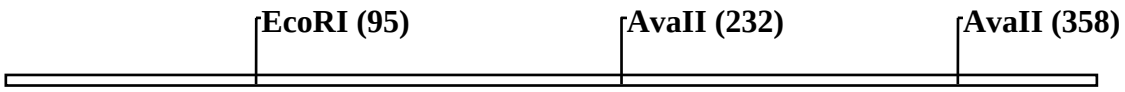
EcoRI + AvaII

Flannelmouth sucker (*Catostomus latipinnis*)



Cutters : EcoRI
Non-Cutters : AvaII

Bluehead sucker (*Pantosteus discobolus*)



Cutters : AvaII & EcoRI

Razorback sucker (*Xyrauchen texanus*)

Non-Cutters : AvaII & EcoRI

Appendix I. Larval fishes examined for genetic variation at two microsatellite loci. Genotypes at each microsatellite locus are reported as the length of each allele. For example, a genotype score of 157159 indicates a heterozygote with allele 1 length = 157 bp and allele 2 length = 159 bp. Zero indicates missing data. Field numbers correspond to field notes accessioned in the Museum of Southwestern Biology Division of Fishes.

Collection Locality (RM = river mile)	mtDNA D-Loop*	Microsatellite Genotype		Species ID	Field No.
		RBS 5H2	RBS 2H2		
San Juan RM 13.0	CL	157159	308308	*C. latipinnis*	WHB02-051
San Juan RM 133.7		161161	308310	*C. latipinnis*	WHB02-061
San Juan RM 115.5		157159	308308	*C. latipinnis*	WHB02-071
San Juan RM 11.5	CL	161161	308312	*C. latipinnis*	WHB02-109
San Juan RM 41.8	CL	161161	308308	*C. latipinnis*	WHB02-196
San Juan RM 126.0		149149	308308	*C. latipinnis*	WHB02-065
San Juan RM 126.0		149157	308308	*C. latipinnis*	WHB02-065
San Juan RM 126.0		0	308308	*C. latipinnis*	WHB02-065
San Juan RM 126.0		0	308308	*C. latipinnis*	WHB02-065
San Juan RM 126.0		151155	308308	*C. latipinnis*	WHB02-065
San Juan RM 126.0		155155	302302	*C. latipinnis*	WHB02-065
San Juan RM 131.8		151151	308310	*C. latipinnis*	WHB02-219
San Juan RM 126.0	PD	149167	228228	*P. discobolus*	WHB02-065
San Juan RM 126.0	PD	149149	228228	*P. discobolus*	WHB02-065
San Juan RM 126.0	PD	0	228228	*P. discobolus*	WHB02-065
San Juan RM 126.0	PD	147147	228228	*P. discobolus*	WHB02-065
San Juan RM 126.0	PD	155155	228228	*P. discobolus*	WHB02-065
San Juan RM 126.0		149151	228228	*P. discobolus*	WHB02-065
San Juan RM 126.0		149155	228228	*P. discobolus*	WHB02-065
San Juan RM 126.0		0	228228	*P. discobolus*	WHB02-065
San Juan RM 126.0		149155	228228	*P. discobolus*	WHB02-065
San Juan RM 126.0		157163	228228	*P. discobolus*	WHB02-065
San Juan RM 126.0		149167	228228	*P. discobolus*	WHB02-065
San Juan RM 126.0		155159	228288	*P. discobolus*	WHB02-065
San Juan RM 126.0		157157	228228	*P. discobolus*	WHB02-065
San Juan RM 126.0		137137	228228	*P. discobolus*	WHB02-065
San Juan RM 126.0		0	228228	*P. discobolus*	WHB02-065
San Juan RM 126.0		155155	228228	*P. discobolus*	WHB02-065
San Juan RM 126.0		149149	228228	*P. discobolus*	WHB02-065
San Juan RM 126.0		163173	228228	*P. discobolus*	WHB02-065
San Juan RM 126.0		155163	228228	*P. discobolus*	WHB02-065
San Juan RM 126.0		0	228228	*P. discobolus*	WHB02-065
San Juan RM 126.0		0	228228	*P. discobolus*	WHB02-065
San Juan RM 126.0		149149	228228	*P. discobolus*	WHB02-065
San Juan RM 5.2		147163	228228	*P. discobolus*	WHB02-065
San Juan RM 85.5		157157	228228	*P. discobolus*	WHB02-055
San Juan RM 139.5		155155	228288	*P. discobolus*	WHB02-084
San Juan RM 100.6		157157	228228	*P. discobolus*	WHB02-058

Appendix I.
continued

Collection Locality		Microsatellite Genotype		Species ID	Field No.
		RBS 5H2	RBS 2H2		
San Juan RM 56.6		147147	228228	*P. discobolus*	WHB02-077
San Juan RM 56.6		167167	228228	*P. discobolus*	WHB02-151
San Juan RM 126.0		159167	228228	*P. discobolus*	WHB02-151
San Juan RM 126.0		155155	228228	*P. discobolus*	WHB02-065
San Juan RM 126.0		0	228228	*P. discobolus*	WHB02-065
San Juan RM 126.0		169177	228228	*P. discobolus*	WHB02-065
San Juan RM 126.0		0	228228	*P. discobolus*	WHB02-065
San Juan RM 126.0		0	228228	*P. discobolus*	WHB02-065
San Juan RM 126.0		0	228228	*P. discobolus*	WHB02-065
San Juan RM 126.0		0	228228	*P. discobolus*	WHB02-065
San Juan RM 126.0		0	228228	*P. discobolus*	WHB02-065
San Juan RM 126.0		157159	228228	*P. discobolus*	WHB02-065
San Juan RM 139.5	PD	0	228228	*P. discobolus*	WHB02-058
San Juan RM 11.5					
San Juan RM 11.5	XT	157157	320320	*X. texanus*	WHB02-109
San Juan RM 71.3	XT	157157	320320	*X. texanus*	WHB02-109
San Juan RM 8.7	XT	157157	320320	*X. texanus*	WHB02-091
San Juan RM 11.6		0	320320	*X. texanus*	WHB02-054
San Juan RM 11.6		157157	320320	*X. texanus*	WHB02-052
San Juan RM 62.3		0	320320	*X. texanus*	WHB02-052
San Juan RM 62.3	XT	149159	320320	*X. texanus*	WHB02-148
San Juan RM 74.9	XT	153153	320320	*X. texanus*	WHB02-142
San Juan RM 126.0		0	228308	*P. d. x C. l.*	WHB02-065
San Juan RM 126.0		0	228308	*P. d. x C. l.*	WHB02-065

*MtDNA d-loop key CL = *C. latipinnis* pattern; PD = *P. discobolus* pattern; XT = *X. texanus* pattern – see Figure 3 for description of restriction fragment patterns.

Appendix II. Adult/Juvenile fishes examined for genetic variation at genetic markers. Genotypes at each microsatellite locus are reported as the length of each allele in base pairs. Zero indicates missing data. Field numbers correspond to field notes accessioned in the Museum of Southwestern Biology Division of Fishes. A source locality is reported for samples with no associated field note.

	MtDNA	Microsatellite Genotype		Field no./Source
Species ID	D-Loop	RBS 5H1	RBS 2H2	
C. latipinnis	PD	149149	310316	DLP4961
C. latipinnis		153161	308308	DLP4961
C. latipinnis		149149	308308	DLP4961
C. latipinnis		161161	308308	DLP4961
C. latipinnis	CL	151151	310312	DLP4962
C. latipinnis		149149	308308	DLP4962
C. latipinnis		0	308308	DLP4962
C. latipinnis		149149	308308	DLP4962
C. latipinnis		149149	308308	DLP4962
C. latipinnis		143149	308308	DLP4962
C. latipinnis		0	308308	DLP4962
C. latipinnis		149149	308308	DLP4962
C. latipinnis		149149	308308	DLP4962
C. latipinnis		149149	300308	DLP4962
C. latipinnis		149149	308308	DLP4962
C. latipinnis		149149	308308	DLP4962
C. latipinnis	CL	0	308318	DLP4962
C. latipinnis		0	308308	DLP4963
C. latipinnis		149149	308308	DLP4964
C. latipinnis		149149	308310	DLP4964
C. latipinnis		137137	308308	DLP4964
C. latipinnis		157157	308308	DLP4964
C. latipinnis		151151	308308	DLP4964
C. latipinnis	CL	149159	308318	DLP4964
C. latipinnis		151151	308312	DLP4964
C. latipinnis		149149	308308	DLP4964
C. latipinnis		149149	310310	DLP4961
C. latipinnis		151000	308312	DLP4964
P. discobolus		147167	228288	DLP4961
P. discobolus		155167	228288	DLP4961
P. discobolus		167179	228228	DLP4961
P. discobolus		147147	228228	DLP4961
P. discobolus		167167	228228	DLP4961
P. discobolus		0	228228	DLP4961
P. discobolus		163169	228288	DLP4962
P. discobolus		147169	228228	DLP4964
P. discobolus		167167	228288	DLP4964
P. discobolus		145159	228228	DLP4964
P. discobolus		0	288288	DLP4967

Appendix II. cont.

Species ID	MtDNA D-Loop	Microsatellite Genotype		Field no./Source
		RBS 5H1	RBS 2H2	
P. discobolus		0	228228	DLP4967
P. discobolus		0	228228	DLP4968
P. discobolus		0	228228	DLP4968
P. discobolus		0	228228	DLP4970
P. discobolus		0	228288	DLP4970
P. discobolus		0	228288	DLP4970
P. discobolus		0	228228	DLP4973
P. discobolus		0	288288	DLP4973
P. discobolus		0	228228	DLP4974
X. texanus	XT	161000	318318	Willow Beach NFH
X. texanus	XT	159163	316320	Willow Beach NFH
X. texanus	XT	153157	320320	Willow Beach NFH
X. texanus	XT	163167	320320	Willow Beach NFH
X. texanus	XT	0	306306	Willow Beach NFH
X. texanus		157157	314314	Dexter NFH
X. texanus		157157	314320	Dexter NFH
X. texanus	XT	157157	314320	Dexter NFH
X. texanus		157157	320320	Dexter NFH
X. texanus	XT	153153	320320	Dexter NFH

*MtDNA d-loop key CL = *C. latipinnis* pattern; PD = *P. discobolus* pattern; XT = *X. texanus* pattern – see Figure 3 for description of restriction fragment patterns. The mtDNA d-loop was sequenced completely for all razorback suckers listed in Appendix II.

Appendix III. Detailed Molecular Methods.

Genomic DNA was isolated from air-dried ethanol-preserved tissues by digesting tissue (whole individual larva or fin clips) in 200 μl STE, 10 μl 20% SDS, and 4 μl proteinase K (10 μg/ml stock) at 55^0C for at least four hours. Samples were then extracted one time with 200 μl PCI (25 parts phenol: 24 parts chloroform: 1 part isoamyl alcohol), once with 200 μl CI (24 parts choloroform: 1 part isoamyl alcohol), and precipitated in 500 μl of 100% EtOH and 20 μl of 2 M NaCl. Following centrifugation, nucleic acids were air-dried were then resuspended in 50 μl of sterile distilled water.

Two single-copy nuclear (microsatellite) loci, RBS-5H1 and RBS-2H2 (Turner et al., unpublished) were also amplified by PCR. Amplification occurred in duplex under the following conditions: 5-10 ng DNA per μl (final reaction volume), 1x PCR buffer, 200 μM each dGTP, dATP, dTTP, dCTP, 1.5 mM $MgCl_2$, 2.5 μm of each primer, and 0.5 units of *Taq* DNA polymerase. One primer of each primer pair was tagged with a fluorescein marker and PCR products were visualized using the ABI 377 automated sequencer, and scored using GeneScan software (Applied Biosystems). Alleles were scored according to their length measured in base pairs (bp) by incorporating appropriate size standards in the gel.

The control region or D-loop of the mitochondrial genome was amplified by PCR with the primers DL 9 (5'-TCAAAGCTTACACCAGTCTTGTAAACC) and DL 12 (5'-CCTGAACTAGGAACCAGATG). Amplification occurred under the following conditions: 5-10 ng DNA per μl (final reaction volume), 1x PCR buffer, 200 μM each dGTP, dATP, dTTP, dCTP, 1 mM $MgCl_2$, 5 μm of each primer, and 0.5 units of *Thermus aquaticus* (*Taq*) DNA polymerase. To obtain D-loop sequence, 50 μl PCR reactions were conducted using 5 μl of product from 10 μl PCRs under the same conditions as described above except that primer concentration was reduced to 0.5 uM. The reactions were then cleaned using the Rapid PCR Purification System (Marligen Biosciences) and eluted in 40 μl of distilled water. 50-100 ng of product were then used as template for nucleotide sequencing using the Big Dye™ reaction kit (Applied Biosystems), following manufacturer's instructions. Nucleotide sequence was obtained from three individuals of each species. Sequencher v. 3.1 (Gene Codes Corporation) was used to align nucleotide

Appendix III. cont.

sequences and to determine that a combination of the restriction enzymes EcoR1 and AvaII could be used to distinguish D-loop sequences from each species (Figure 1). For remaining individuals, D-loop was amplified in 10 μl reactions and the product was digested for 2 hours at 37^{o}C in 20 μl volume reactions under the following conditions: 7 μl PCR product, 1x buffer (provided by the manufacturer), and 5 units of enzyme. The PCR products were visualized on an ethidium bromide-stained 0.8% agarose gel, both before and after restriction enzyme digestion.