

Mitochondrial DNA Analysis of Burbot Stocks in the Kootenai River Basin of British Columbia, Montana, and Idaho

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Abstract.—Differences in mitochondrial haplotype frequency were examined among burbot *Lota lota* collected from four areas within the Kootenai River Basin of British Columbia, Montana and Idaho. The polymerase chain reaction (PCR) was used to amplify three gene regions of the mitochondria genome: NADH dehydrogenase subunit 1 (ND1), NADH dehydrogenase subunit 2 (ND2), and NADH dehydrogenase subunits 5 and 6 combined (ND5,6). Amplified DNA was screened for restriction fragment length polymorphisms (RFLPs). Simple haplotypes resulting from RFLPs in a single gene region were combined into composite haplotypes. The distribution of composite haplotypes and their frequencies correspond to areas of the Kootenay River basin above and below a presumptive geographic barrier, Kootenai Falls, Montana, and suggest spatially segregated populations. A test of geographic heterogeneity among haplotype frequency distributions was highly significant ($P < 0.001$) when a Monte Carlo simulation was used to approximate a X^2 test. Two populations, one above and one below Kootenai Falls emerged when a neighbor-joining method was used to infer a phylogenetic tree based on estimates of nucleotide divergence between all pairs of sample locations. These analyses indicate that burbot below Kootenai Falls form a separate genetic group from burbot above the falls and further suggests that Libby Dam, which created Lake Kootenay, is not an effective barrier segregating burbot above Kootenai Falls. Management implications from these findings are that mitigative efforts in the Kootenai River Basin must address the needs of two genetically divergent burbot stocks, and that a unification of angling regulations for the population downstream of Kootenai Falls in Idaho and British Columbia is warranted.

Burbot *Lota lota* are benthic, freshwater members of the cod family (Gadidae) with a Holarctic distribution; in North America, their range includes a majority of mainland Canada and several northern U.S. states (Scott and Crossman 1973). Burbot are endemic to the Kootenai River basin which, includes Kootenay Lake, British Columbia, and extends through the northern reaches of Idaho and Montana in the United States. Burbot once provided popular sport fisheries within the drainage (Simpson and Wallace 1982; Paragamian 1993) and produced up to 26,000 fish annually in Kootenay Lake (Andrusak 1976). In general, burbot numbers in the Kootenai River have declined since 1959 (Partridge 1983). However, since construction of a hydropower and flood control dam by the U.S. Army Corps of Engineers (USACE) on the Kootenai River near Libby, Montana, in 1972, the burbot fisheries in the Kootenai River

in Idaho and in Kootenay Lake have collapsed (Paragamian 1993). The collapse of burbot fisheries is not fully understood but is thought to be partially due to changed Kootenai River discharge patterns during the winter spawning season, elevated winter temperatures, and reduced primary and secondary productivity (Paragamian et al., in press). At present, burbot fisheries in Idaho, Montana, and British Columbia are the subjects of cooperative research to determine factors limiting recruitment. The goal of this program is to restore the burbot population and fishery through management and to request mitigative measures from the USACE.

Telemetry, creel, and tagging data indicate there may be at least two burbot stocks in the Kootenai basin. One putative stock extends from Kootenai Falls, Montana, through Idaho to Kootenay Lake. A second population and possibly a third occur in Montana above Kootenai Falls and above Libby Dam, respectively (Partridge 1983; Paragamian 1995). Data suggest that burbot in Montana are a fluvial stock whereas fish in Kootenay Lake and

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Idaho have a variant adfluvial life history, moving freely between Kootenay Lake and the river to spawn in river tributaries (Paragamian 1995). Consequently, it is important to determine the level of genetic differentiation among these putative stocks because of the management implications of multistock restoration.

In this study, we used restriction fragment length polymorphism (RFLP) analysis of amplified mitochondrial DNA gene regions to determine stock differentiation among presumptive burbot populations in the Kootenai River drainage.

Study Area

The Kootenai basin's 34,490 km² make it the second largest drainage in the Columbia River system. Two natural barriers potentially segregate burbot populations within the Kootenai River and between the Kootenai and Columbia rivers (Figure 1). The downstream barrier is Bonnington Falls, downstream from Kootenay Lake and just above the Columbia River confluence; the upstream barrier is Kootenai Falls, Montana. These natural barriers have been present for approximately 10,000 years (Northcote 1973). Additionally, Libby Dam, completed in 1972 at river kilometer (rkm) 352 from the north end of Kootenay Lake, now forms a potential barrier between burbot populations in Lake Koocanusa and the Kootenai River below the dam.

Methods

Collections.-Tissue samples from 147 burbot were collected from four areas of the Kootenai River basin (Figure 1). In Idaho and British Columbia, burbot were captured in Kootenay Lake (location KL; $N = 13$) and in rkm 145-245 of the Kootenai River (area ID, $N = 80$). In Montana burbot were captured near the base of Libby Dam (area MT, $N = 20$) and from rkm 352 to 44 above Libby Dam in Lake Koocanusa (area LK, $N = 34$). The samples represent three putative burbot populations: (1) Kootenay Lake and Kootenai River below Kootenai Falls; (2) Kootenai River above Kootenai Falls and below Libby Dam; and (3) Lake Koocanusa formed by Libby Dam. Burbot were caught with baited hoop nets of two sizes (3.1 m and 3.7 m long) from November 1994 through February 1998 (Bernard et al. 1991). Chunks of cut fish were placed in a woven bait bag and suspended from the second to last hoop (from the entrance) inside each net. Burbot captured in the hoop nets were measured in total length and weighed individually. Each burbot was

tagged with a passive integrated transponder tag in the cheek muscle, a sample of its fin was taken for genetic analysis, and it was released.

Analysis.-Fin samples from each burbot were stored in 70% ethanol until DNA was extracted by methods modified from Sambrook et al. (1989) and Dowling et al. (1990). Approximately 100 mg of tissue was excised from each sample, transferred to a 1.7- μ L tube, homogenized in 650 μ L of digestion buffer (50 mM tris-HCl, pH 8.0; 200 mM NaCl; 50 mM EDTA; 1% sodium dodecyl sulfate; 0.2% dithiothreitol; and 0.5% proteinase K), and incubated overnight at 55°C. Following overnight digestion, the samples were extracted twice with equal volumes of chloroform-isoamyl alcohol, the aqueous phase was removed, and DNA was precipitated with 0.1 volume of 3 M ammonium acetate and 2 volumes of cold 100% ethanol. DNA was centrifuged at 13,000 revolutions per minute in a microfuge for 10 min and the resulting pellet was washed twice in 500 μ L of 70% ethanol, dried at 37°C, and resuspended in 100 μ L of 1 X tris-EDTA buffer.

The polymerase chain reaction (PCR) was used to amplify sequences with a thermocycler profile of 95°C for 45 s, 48°C for 40 s, and 70°C for 150 s run for 38 cycles, followed by 180 s at 70°C. Nucleotide primers specific for the mitochondrial NADH dehydrogenase (ND) subunits 1, 2, and combined 5 and 6 (LGL Ecological Genetics) were used in a 40 μ L reaction volume containing 1-3 μ L of sample DNA, 16 pmol of primers, 1 mM of deoxynucleotide triphosphates, 0.5 units of *Thermus aquaticus* (*Taq*) DNA polymerase, and 1X reaction buffer supplied by the manufacturer (Perkin-Elmer Corp.). Primers were generally designed for fish mitochondrial DNA and amplified well for burbot (subunit ND1: primer 381, 5'-ACC CCG CCT GTT TAC CAA AAA CAT 3', and primer 563-B, 5'-GGT TCA TTA GTG AGG GAA GG-3'; subunit ND2: primer 562, 5'-TAA GCT ATC GGG CCC ATA CC-3', and primer 461, 5'-GGC TCA GGC ACC AAA TAC TAA-3'; subunits ND5,6: primer 763, 5'-AAT AGC TCA TCC ATT GGT CTT AGG-3', and primer 764, 5'-TAA CAA CGG TGG TTT TTC AAG TCA-3'). Amplified mitochondrial DNA (mtDNA) gene regions were digested with four restriction endonucleases: *Hae* III, *Dpn* II, *Rsa* I, and *Taq* I (New England Biolabs). The resulting mtDNA fragments were separated by electrophoresis in gels of 3% agarose and tris acetate-EDTA buffer. Vertical 6% polyacrylamide gels with tris-borate-EDTA were used to separate small fragments and questionable co-

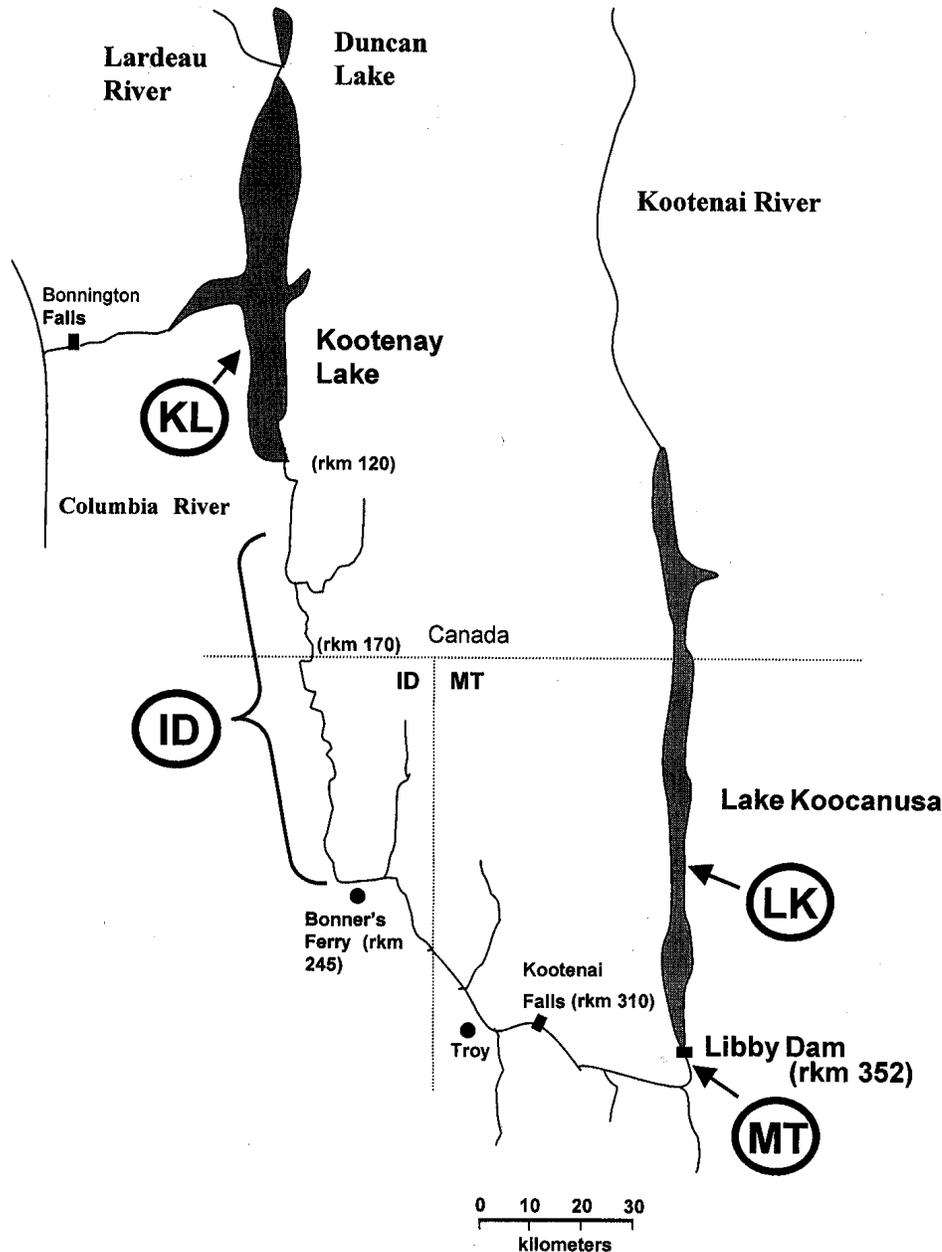


FIGURE I.-Map of the Kootenai River basin of British Columbia (Canada), Montana (MT), and Idaho (ID) showing notable points, river kilometers (rkm) from the north end of Kootenay Lake, and burbot collection areas (circled abbreviations).

migrating fragments. Gels were stained with ethidium bromide and restriction fragment patterns were visualized in ultraviolet light. Photographs of each gel were converted into computer image files via a ScanMan scanner and ScanMan 2.0 software (Logitech). Restriction fragment length polymorphisms (RFLPs) observed among samples were visualized with SigmaScan Pro 3.0 (Jandel

Scientific 1996) and then given alphabetical designations as haplotypes. The size of each DNA fragment from each mtDNA gene region was estimated by comparison to a size standard, the pUC-19 marker (Bio-Synthesis). Alphabetical designations for RFLPs of each mtDNA gene region were combined into composite mtDNA haplotypes. An estimate of the number of nucleotide

TABLE 1.-Composite mtDNA haplotypes of NADH dehydrogenase and their frequencies (in parentheses) observed among 147 burbot from four sample areas (Figure 1) in the Kootenai River basin. Simple haplotypes are combined fragment length patterns in digested mtDNA.

Composite haplotype and sample <i>N</i>	Simple haplotype	Sample area				Haplotype <i>N</i>
		KL	ID	MT	LK	
Bur-01	AAAAAA	11 (0.846)	51 (0.638)	6 (0.300)	8 (0.235)	76 (0.517)
Bur-02	AAAABA	0	8 (0.100)	11 (0.550)	23 (0.676)	42 (0.286)
Bur-03	AAAAEA	0	18 (0.225)	0	0	18 (0.122)
Bur-04	AAABAA	0	0	2 (0.100)	1 (0.029)	3 (0.020)
Bur-05	BAAAAA	0	0	1 (0.050)	1 (0.029)	2 (0.014)
Bur-06	CAAAAA	2 (0.154)	0	0	0	2 (0.014)
Bur-07	AAAACA	0	1 (0.013)	0	0	1 (0.007)
Bur-08	AAAADA	0	1 (0.013)	0	0	1 (0.007)
Bur-09	AAACBA	0	0	0	1 (0.029)	1 (0.007)
Bur-10	ABAACA	0	1 (0.013)	0	0	1 (0.007)
<i>N</i>		13	80	20	34	147

substitutions per site (*p*) for each RFLP was calculated via the Nei (1987) method and REAP 4.0 (Restriction Enzyme Analysis Package) (McElroy et al. 1991) and then used to generate a matrix comparing *p* values (distance) between all pairs of identified composite haplotypes. The KITSCH program in PHYLIP 3.5 (Felsenstein 1993), which assumes independence and equal rates of divergence, was then used to generate a distance dendrogram by the least-squares method of Fitch and Margoliash (1967) to illustrate the estimated evolutionary relationships and distance among the identified composite haplotypes. The significance of geographic heterogeneity among population frequency distributions of composite haplotypes was assessed via Monte Carlo simulation as described by Roff and Bentzen (1989), for which the MONTE program in REAP 4.0 was used (McElroy et al. 1991) with 1,000 randomizations. The frequency distribution of composite haplotypes and the estimates of evolutionary distance were employed to construct a modified matrix of nucleotide divergence among all sample locations via the DA program in REAP 4.0 (McElroy et al. 1991). This matrix was then employed to infer a phylogenetic tree by applying the neighbor-joining method (Saitou and Nei 1987; Swofford and Olsen 1990) incorporated in NTSYS-pc 1.80 (Numerical Taxonomy and Multivariate Analysis System) (Rohlf 1993).

Results

Variation in three mtDNA gene regions among burbot samples from four areas revealed 10 composite mtDNA haplotypes (henceforth haplotypes) designated Bur-01 through Bur-10 (Table 1). Variation among haplotypes Bur-01, Bur-02, Bur-03,

Bur-07, and Bur-08 are due to polymorphisms observed in the amplified ND5,6 gene region digested with *Rsa I*. Haplotypes Bur-05 and Bur-06 differ by a polymorphism in *Dpn II* digests of the ND1 region. Haplotype Bur-04 differs from Bur-01 by a polymorphism in the ND2 region digested with *Rsa I*. Haplotype Bur-09 differs from Bur-01 due to observed polymorphisms in *Rsa I* digests in the amplified ND2 and ND5,6 regions. Haplotype Bur-10 differs from Bur-01 due to a polymorphism in *Hae III* digests of the ND1 region and *Rsa I* digests of the ND5,6 region. Observed haplotypes followed a general pattern. Each area sample contained two or three haplotypes that accounted for a majority of individuals (91.1-100.0%) and all samples contained minor haplotypes (those with frequencies $\leq 5.0\%$) except Kootenay Lake (KL).

Haplotype Bur-01 was observed in all four samples and increased in frequency from 24% in Lake Kootenay (LK) downstream to 85% in Kootenay Lake. Concurrently, Bur-02 decreased in frequency from 66% in Lake Kootenay downstream to 0% Kootenay Lake. Three minor haplotypes were observed in Lake Kootenay: Bur-04 and Bur-05, shared with Kootenay River, Montana (MT), samples, and Bur-09, which was only observed in Lake Kootenay. The Kootenay River, Idaho-British-Columbia (ID) sample contained four haplotypes not observed in the other locations: Bur-03 with a frequency of 23% and the minor haplotypes Bur-07, Bur-08, and Bur-10. Only two haplotypes were observed in Kootenay Lake: Bur-01 and Bur-06. The estimated percent sequence divergence among haplotypes within each sample varied from 0.0385 to 0.1933 (Table 2). The estimated percent sequence divergence among haplotypes between lo-

TABLE 2.-Percent sequence divergence ($p \times 100$) among 10 composite mtDNA haplotypes observed in burbot populations from the Kootenai River basin. Composite haplotype designations are from Table 1.

Haplotype	Composite haplotype								
	Bur-01	Bur-02	Bur-03	Bur-04	Bur-05	Bur-06	Bur-07	Bur-08	Bur-09
Bur-02	0.0656								
Bur-03	0.0656	0.1014							
Bur-04	0.0385	0.1214	0.1214						
Bur-05	0.0841	0.1933	0.1933	0.1399					
Bur-06	0.0385	0.1214	0.1214	0.0841	0.0841				
Bur-07	0.0385	0.0656	0.0656	0.0841	0.1399	0.0841			
Bur-08	0.1014	0.0796	0.0796	0.1733	0.2747	0.1733	0.1014		
Bur-09	0.0841	0.0200	0.1214	0.0841	0.2188	0.1399	0.0841	0.1014	
Bur-10	0.1014	0.1399	0.1399	0.1572	0.2291	0.1572	0.0558	0.1933	0.1572

cations was highest at 0.2747 between Bur-05, shared by the MT and LK samples, and Bur-08 in the ID sample.

The distance dendrogram indicates presumptive relationships among observed haplotypes (Figure 2). The two most frequently observed haplotypes among sample locations, Bur-01 and Bur-02, do not cluster closely together. Haplotypes Bur-04 and Bur-05, shared by MT and LK samples are also dissimilar. Minor haplotypes Bur-09 (LK) and Bur-05 (LK and MT) are dissimilar and do not cluster closely with the other haplotypes. Conversely, haplotypes Bur-02 and Bur-03 cluster together, as do Bur-07 with Bur-08 and Bur-06 with Bur-10.

The neighbor-joining, unrooted tree of inferred

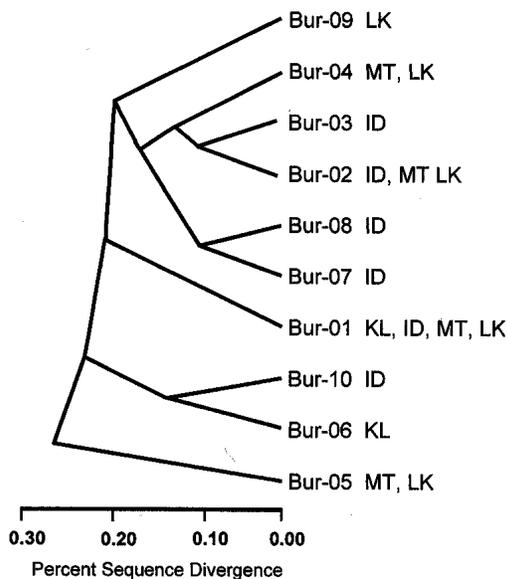


FIGURE 2.-Dendrogram of 10 composite mtDNA haplotypes (Bur; Table 1) observed among 147 burbot sampled from four areas (Figure 1) in the Kootenai River basin.

phylogenetic relationships shows the two locations above Kootenai Falls clustering together and distinct from the two sample locations below the falls (Figure 3). Geographic heterogeneity among frequency distributions of haplotypes was highly significant ($X^2=102.05$; $P < 0.001$) in the Monte Carlo simulation with 1,000 randomizations. Thus, the null hypothesis of no significant geographic variation in haplotype distribution was rejected.

Discussion

Brown (1983) indicated that mitochondrial DNA generally evolves at a rate of approximately 2% per million years. Although this rate is rapid compared to the evolution of known nuclear gene regions, it is still too slow to allow for a great deal of nucleotide divergence between populations that have become isolated only within the past 10,000 years. Even with complete isolation, populations that have colonized habitats since the Pleistocene will show little genetic divergence simply based on mutation (Billington and Hebert 1991). Thus, it is unreasonable to expect burbot stocks above and below Kootenai Falls to be differentiated solely by unique haplotypes that have arisen through mutation since their recent separation. However, genetic information from mtDNA can still be a sensitive measure of population divergence (Birky et al. 1983) and thus mtDNA can be used in other ways for stock discrimination within the geographic and temporal scale of the Kootenai River drainage. First, nucleotide divergence can still be observed between recently separated populations. However, it is important to realize that this divergence is most likely a result of sampling effects associated with population founding (Billington and Hebert 1991). Secondly, shifts in mtDNA haplotype frequency are commonly used to discriminate stocks and have been used in a number of studies of other fish species including Chinook

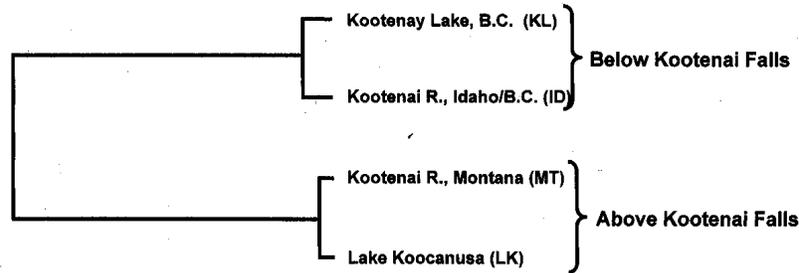


FIGURE 3.-Inferred phylogenetic relationships among burbot from four sample areas in the Kootenai River basin constructed by using the neighbor-joining method of Saitou and Nei (1987).

salmon *Oncorhynchus tshawytscha* (Wilson et al. 1987), American shad *Alosa sapidissima* (Bentzen et al. 1989), and white perch, *Morone americana* (Mulligan and Chapman 1989).

Both the sequence divergence among observed haplotypes and the highly significant geographic heterogeneity observed in haplotype frequency distributions support a conclusion of two genetically dissimilar burbot populations. The results of the present mtDNA RFLP analyses are also supported by earlier tagging and telemetry studies that indicated burbot in Kootenay Lake and the Kootenai River in Idaho and British Columbia may be the same stock (Paragamian 1995). In addition, none of more than 400 burbot that have been tagged in Montana have been recaptured in Idaho or British Columbia (Greg Hoffman, Montana Department of Fish, Wildlife, and Parks, personal communication), whereas 12 of 145 burbot tagged in the Idaho-British Columbia stretch of the Kootenai River have been recaptured in that reach (file data). Burbot move freely between the river and lake and after a prespawning migration, spawn in tributaries to the river (Paragamian 1995).

The frequency distributions of haplotypes Bur-01 and Bur-02 apparently change sharply at Kootenai Falls, where Bur-01 being the most common haplotype in samples from below the falls and Bur-02 the most common in upstream samples. However, studies have shown burbot tagged in Idaho do not move upstream further than rkm 246 (Paragamian 1995). Moreover, previous sampling from Bonners Ferry, Idaho (rkm 245; Figure 1), to the Idaho-Montana border (rkm 276) indicated burbot were nearly nonexistent there (Paragamian 1993). The habitat degradation that has occurred in this reach of the Kootenai River within the last 25 years may have added an anthropogenic barrier to the natural barrier of Kootenai Falls to further isolate burbot populations. Further, the ID area downstream of rkm 245 contains relatively few burbot;

the large ID sample for our study resulted from several years of fishing effort.

Studies in Montana have shown that burbot in Lake Koocanusa are entrained through Libby Dam (Skarr et al. 1996). Telemetry of burbot in Lake Koocanusa suggests a spawning run through the unregulated river as far upstream as rkm 475 (Scott Snelson, Montana Department of Fish Wildlife, and Parks, personal communication). The results of the present study suggest the burbot sampled just below Libby Dam are genetically similar to those found in Lake Koocanusa. Fish from both areas share four of five haplotypes, including two minor haplotypes, and the two samples cluster together (Figure 3). Thus, as a potential artificial barrier, Libby Dam is evidently not a significant deterrent to the downstream movement of burbot above Kootenai Falls.

Implementation of a water management program by the USACE to increase the number of burbot in Montana may not improve the burbot fishery in Idaho and British Columbia. It will be important to characterize the life histories of both burbot populations so that resource managers can determine which hydrologic mitigative measures are compatible with populations both above and below Kootenai Falls. Perhaps management and recovery programs will have to differ between the two populations. The recovery of one population should not justify neglect of the second.

Because the burbot in Kootenay Lake and the Kootenai River of Idaho and British Columbia are genetically similar, uniform management regulations are appropriate and should be consistent for this region. The fisheries in Idaho and British Columbia are currently closed. Burbot are also found in the north arm of Kootenay Lake and particularly in the Duncan and Lardeau rivers, and they may represent additional stocks. It will be worthwhile to extend mtDNA RFLP analysis to these fish to

ascertain their uniqueness and need for special management attention.

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