

1 Ancestry, population structure, and conservation genetics of Arctic grayling (*Thymallus arcticus*)  
2 in the upper Missouri River, USA

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17 Revision 1: April 23, 2009

18 Accepted: June 17, 2009

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19 **Abstract**

20 We genotyped Arctic grayling (*Thymallus arcticus*) at 10 microsatellite loci in 18 samples (n =  
21 726) from Montana, Wyoming, and Saskatchewan to determine genetic relationships among  
22 native, captive, and naturalized populations in the upper Missouri River basin; assess patterns in  
23 genetic diversity; and infer recent demographic histories. Substantial genetic subdivision was  
24 observed among sample populations (global  $F_{ST}=0.10$ ). Canadian populations have been isolated  
25 from Missouri River populations long enough for mutation to have caused genetic differences  
26 between regions (mean pairwise  $F_{ST} = 0.18$ ,  $R_{ST} = 0.54$ ). Within the Missouri River basin, most  
27 naturalized lacustrine populations traced their ancestry to Red Rock lakes. Two populations in  
28 headwater lakes within the Big Hole River watershed appear to be native. We found no evidence  
29 for introgression of Canadian-origin grayling, nor any effect of hatchery stocking in native  
30 populations. The native fluvial Big Hole River group was genetically distinct and most diverse  
31 ( $H_E = 0.89$ ), whereas native Madison River and Red Rock lakes populations exhibited lower  
32 genetic diversity ( $H_E = 0.74$  and  $0.80$ , respectfully) and evidence of recent bottlenecks. The  
33 existing Big Hole and Red Rock populations are at low abundance but do not appear to be at  
34 immediate risk of inbreeding depression ( $N_e$  207.7-228.2).

35

36 Keywords: bottleneck, habitat fragmentation, hatchery stocking, microsatellites, population  
37 subdivision

## 38 **Introduction**

39           Understanding population-level consequences of patterns in neutral genetic variation has  
40 become a preliminary step in the development of conservation programs for many commercially-  
41 exploited or sensitive species. In addition to helping determine ancestral relationships and  
42 phylogeny, these patterns are commonly used to infer genetic connectivity among demes and  
43 help define populations (Waples and Gaggiotti 2006), which are the logical focus of conservation  
44 and management (Morris and Doak 2002). Microsatellite DNA markers can help identify cryptic  
45 population structure (e.g., So et al. 2006), and reconstruct demographic histories for rare species  
46 (e.g., Nielsen et al. 1999). Genetic-based methods can both characterize and distinguish between  
47 historical and contemporary factors that influence the demographic histories of extant  
48 populations (Pearse and Crandall 2004), for example glacial events across tens of thousands of  
49 years (Bernatchez and Wilson 1998) versus recent habitat fragmentation, human exploitation,  
50 nonnative species invasions, or other impacts over tens or hundreds of years (e.g., Koskinen et al.  
51 2002a; Stamford and Taylor 2005). These properties make microsatellite markers particularly  
52 amenable to understanding the conservation status of northern freshwater fishes, like Arctic  
53 grayling, (*Thymallus arcticus*), that have been strongly influenced by both types of factors.

54           Arctic grayling are stenothermic cold-water, salt-water intolerant salmonids. They are  
55 native to Arctic Ocean drainages of Alaska and northwestern Canada, as far east as Hudson's  
56 Bay, and westward across northern Eurasia to the Ural mountains (Scott and Crossman 1973;  
57 Froufe et al. 2005; Weiss et al. 2006). In North America, they are also native to northern Pacific  
58 Ocean drainages as far south as the Stikine River in British Columbia. To the south and east of  
59 the main species distribution, two disjunct groups of Arctic grayling are native to the  
60 coterminous United States: one in Michigan (extirpated in the late 1930s, Hubbs and Lagler

61 1949), and one in the upper Missouri River basin in Montana and Wyoming (extant). The  
62 Michigan and Missouri River populations were isolated from northern drainages by Wisconsin  
63 glaciation (Pielou 1991), but the latter apparently began to diverge genetically before that time  
64 (i.e., >70 000 yr ago, Redenbach and Taylor 1999). Glacial history and genetic data suggest the  
65 Missouri River population was founded from individuals that survived Pleistocene glacial  
66 advance in a refuge in the upper Missouri River system or southwestern Alberta, or in both  
67 places (Redenbach and Taylor 1999; Stamford and Taylor 2004).

68 The historical distribution of Arctic grayling in Wyoming and Montana was thought to  
69 encompass approximately 2000 km of lotic habitat in the upper Missouri River system above the  
70 Great Falls (Kaya 1992). A migratory, river-dwelling (fluvial) life history presumably  
71 predominated based on historical reports of Arctic grayling in the main-stem Missouri River and  
72 its major tributaries (e.g., Madison, Jefferson, and Gallatin rivers), and the paucity of lakes  
73 accessible for colonization (Vincent 1962; Kaya 1992). The current distribution of native Arctic  
74 grayling in the Missouri River system is only a fraction (<5%) the historical estimate (Kaya  
75 1992). The combined effects of overexploitation, dams, stream dewatering, and interaction with  
76 nonnative trout species are suspected in this decline (Vincent 1962; Kaya 1992, 2000).  
77 Currently, native Arctic grayling occur only in southwestern Montana at three locations: (i) the  
78 Big Hole River and perhaps two small lakes in that drainage; (ii) Red Rock lakes, which are two  
79 small natural lakes in the headwaters of the Beaverhead River system; and (iii) Ennis Reservoir  
80 on the Madison River. The Big Hole River population has received special conservation focus as  
81 the single-remaining fluvial Arctic grayling population in the coterminous US (Montana Fish,  
82 Wildlife and Parks (MFWP) 1995).

83           Until recently, fluvial Arctic grayling in Montana were considered a candidate species for  
84 listing under the Endangered Species Act (US Fish and Wildlife Service (USFWS) 2007), and  
85 the decision not to extend US federal protection to this population remains controversial.  
86 Nonetheless, conservation efforts continue to focus on improving habitat conditions in the Big  
87 Hole River and re-establishing other fluvial populations in historic waters using fish derived  
88 from the Big Hole River (MFWP 1995, 2007). Ironically, while native Arctic grayling in the  
89 upper Missouri River have declined precipitously in the past 100 yr, culture and stocking of  
90 grayling within and beyond the region has been extensive (Kelley 1931; Kaya 1990; MFWP  
91 2005b). Dozens of naturalized Arctic grayling populations have been established in Montana  
92 lakes (MFWP 2005b). Through the mid-20<sup>th</sup> century, millions of hatchery-reared Arctic grayling  
93 of uncertain origin were planted in Montana waters containing extant native populations,  
94 including the Big Hole River, raising concerns that interbreeding with introduced fish has altered  
95 indigenous gene pools (Everett 1986), which potentially disrupts local adaptation (e.g., Allendorf  
96 et al. 2001).

97           Existing genetic data support the long-term geographic isolation of Arctic grayling from  
98 the upper Missouri River (Everett 1986; Redenbach and Taylor 1999; Stamford and Taylor  
99 2004). These same data provide less insight into ancestral relationships among the extant native  
100 and introduced populations within the Missouri River system. In general, allozymes may exhibit  
101 less variation in recently diverged populations (Hedrick 1999), which may explain why protein  
102 electrophoresis has proven only moderately informative for distinguishing among Arctic grayling  
103 populations in Montana and Wyoming (Everett 1986; R. Leary, University of Montana,  
104 unpublished report, 2005; Campton 2006). The existing mtDNA data are uninformative within  
105 this region as 24 of 25 individuals from the Big Hole River, Red Rock lakes, and Madison River

106 populations had the same composite haplotype (Redenbach and Taylor 1999). In contrast, a  
107 genetic signature of population structure is often detected at the basin or sub-basin scales for  
108 philopatric freshwater fishes like salmonids (e.g., Costello et al. 2003; Hendry et al. 2004).  
109 Indeed, Hop and Gharrett (1989) used electrophoresis to determine population structure of Arctic  
110 grayling in the Chena River, Alaska; and studies of European grayling (*Thymallus thymallus*)  
111 have found significant population structuring within a river system (e.g., Weiss et al. 2002; Gum  
112 et al. 2003; Meldgaard et al. 2003), or even within the same lake (Koskinen et al. 2001). Fine-  
113 scale genetic analyses of North American Arctic grayling using microsatellites are few (see  
114 Stamford and Taylor 2005), but may help answer important conservation questions.

115         Our general objective was to use microsatellite markers to more clearly characterize  
116 ancestral relationships and genetic differentiation among native and naturalized Arctic grayling  
117 populations in the upper Missouri River. Specifically, we used these markers to infer the  
118 genetic origin of introduced populations, to determine if significant admixture between  
119 indigenous and introduced fish has resulted from past stocking, and to characterize the  
120 population structure among native populations at the basin scale (i.e., upper Missouri River). At  
121 the sub-basin scale within the Big Hole River, we sought to establish whether the fluvial  
122 population functions genetically as a single, panmictic population or as a set of demes linked by  
123 occasional gene flow. For the supported native population groupings, we used genetic data to  
124 deduce their demographic histories and identify if, and when, abrupt population declines have  
125 occurred. Results are discussed relative to the genetic status and conservation of native  
126 populations with special emphasis on the Big Hole River, and continuing efforts to restore fluvial  
127 populations in indigenous waters within the upper Missouri River basin.

## 128 **Materials and methods**

129 **Study area and sample collection**

130 We collected samples thought to represent native populations and their derivatives,  
131 introduced populations of Montana or Canadian origin, and a reference population in northeast  
132 Saskatchewan, Canada (Table 1, Figure 1).

Table 1  
near here

133 *Native or putative native populations and derived conservation populations*

Figure 1  
near here

134 From the Big Hole River we obtained Arctic grayling samples from five discrete but  
135 hydrologically connected locations (populations 1-5, Table 1) thought to represent different  
136 demes based on occurrence of young-of-the-year fish (e.g., Liknes and Gould 1987; MFWP  
137 2005a, 2007). Mussigbrod (6) and Miner (7) are samples from small (<50 ha) headwater lakes in  
138 the upper Big Hole River system which are now isolated from the main stem by a diversion dam  
139 and habitat degradation, respectively. Red Rock lakes (8) includes adfluvial grayling collected  
140 during their spawning migration into Red Rock Creek. Madison River-Ennis Reservoir (9) are  
141 grayling collected in or upstream from Ennis Reservoir. This reservoir was formed by  
142 construction of an impassible dam in 1903 (Figure 1).

143 Waters containing populations 1-9 were extensively stocked with grayling, primarily  
144 from the Washoe Park Hatchery (Table 2). Kelley (1931) reported that the Washoe Park  
145 (Anaconda) hatchery and a population in nearby Georgetown Lake were founded with grayling  
146 eggs from Red Rock lakes (Elk Springs Creek) and the Madison River (Meadow Creek). The  
147 Bozeman hatchery population was founded from Red Rock lakes grayling (Kelley 1931).

Table 2  
near here

148 The Ruby River (10), Axolotl Lake (11), and Bozeman Fish Technology Center (12)  
149 samples are derived from Big Hole River grayling taken into captivity for conservation purposes.  
150 The Ruby River has been stocked with captive-spawned juvenile grayling in an attempt to re-  
151 establish a fluvial population (MFWP 2004, 2007). Axolotl Lake is a closed-basin lake that

152 contains a brood reserve established 20 yr ago (MFWP 2007). Grayling (manually) spawned  
153 from Axolotl Lake have been transplanted to the Ruby River and elsewhere in the upper  
154 Missouri River system (MFWP 2007). The Bozeman Fish Technology Center was a hatchery  
155 genetic reserve established between 1988 and 1992 (M. Toner, USFWS, Bozeman, MT, personal  
156 communication).

157 *Introduced and Canadian-origin populations*

158 Odell (13) and Bobcat lakes (16) are a small (<15 ha) headwater lakes in the Wise River  
159 system, a major tributary to the Big Hole River (Figure 1). We could not locate any stocking  
160 records for these locations (Table 1). Grebe Lake (15) is a headwater lake (<40 ha) in the  
161 Madison River system within Yellowstone National Park. Grebe Lake is isolated by a natural  
162 geologic barrier, but grayling were introduced in 1921 and the lake became a major source of  
163 grayling eggs shipped throughout the western US (Kaya 2000). Sunnyslope Canal (17) contains  
164 an introduced population derived from the stocking of its source reservoir between 1937-43  
165 (Barndt and Kaya 2000).

166 Fuse Lake (14) is in the Columbia River basin and contains a population introduced in  
167 1952 from the Mackenzie River system, Canada (Everett 1986; Kaya 1990). We included this  
168 sample to determine if Canadian-origin grayling were further transplanted within the upper  
169 Missouri River. The Fond du Lac (18) area in northeastern Saskatchewan is also part of the  
170 Mackenzie River system, and Fond du Lac grayling were included as a reference population.

171 Samples from most locations consisted of finclips collected from adults representing  
172 multiple cohorts. Native grayling were so rare in the Big Hole and Madison river that samples  
173 were pooled across years (Table 1), and generally included all available individuals, including  
174 young-of-the-year when present. Swamp Creek and Wisdom samples were primarily young-of-

175 the-year from two different cohorts, because adults were not present. Only young-of-the-year  
176 were present at the time of sample collection in Steel Creek, so we systematically selected a sub-  
177 sample of individuals for genotyping to reduce the probability of including full siblings. Scales  
178 were the DNA source for the 1996 Madison River-Ennis Reservoir samples (9) and the entire  
179 Fond du Lac sample (18).

#### 180 **DNA extraction and genotyping of microsatellite loci**

181 Genomic DNA was extracted from a 1 mm<sup>2</sup> piece of fin tissue or a single scale by  
182 placing it in 200 µL of 5% chelex containing 0.2 mg•mL<sup>-1</sup> of proteinase K, incubating it for 2 h  
183 at 56°C, boiling it for 8 min at 100°C, and vortexing it for 30 sec. We used 2 µL of the  
184 supernatant in 15 µL PCR reactions to amplify ten microsatellite loci (Table 3). Conditions for  
185 PCR amplification, primer sequences, and methods for determining multilocus genotypes for all  
186 loci are reported in Diggs and Ardren (2008).

#### 187 **Data analyses**

##### 188 *Within population diversity*

189 Average population gene diversities ( $H_E$ , Nei 1987) and allelic richness for each locus were  
190 estimated using the program HP-RARE 1.0 (Kalinowski 2005). Allelic richness was estimated  
191 using a rarefaction procedure to account for unequal sample sizes (Kalinowski 2004). The allele  
192 permutation test in FSTAT 2.9.3.2 (Goudet 2001) was used to test for differences between groups  
193 of populations in the mean values of allelic richness and  $H_E$ . Conformance of genotypic  
194 frequencies to Hardy-Weinberg expectations (HWE) was evaluated using the methods of Guo  
195 and Thompson (1992) via the program GENEPOP 4.0.7 (Raymond and Rousset 1995). Tests for  
196 genotypic disequilibrium at all pairs of loci in each sample were also calculated using GENEPOP  
197 4.0.7, with 10 000 dememorizations, 10 000 batches and 10 000 iterations per batch. Statistical

198 significance levels used to detect deviations from HWE or genotypic equilibrium ratios were  
199 adjusted for the number of simultaneous tests by the sequential Bonferroni correction (Rice  
200 1989).

201       Sample collections containing close relatives have the potential to bias population genetic  
202 measures of diversity. Many of the samples for this study were collected from populations that  
203 have experienced recent declines thus increasing the chance for the sample containing relatives.  
204 In addition, the tendency for juvenile salmonids to remain in close proximity during their  
205 juvenile life stage often results in the sampling of family groups (Allendorf and Phelps 1981).  
206 We estimated the level of relatedness in each sample by calculating the mean pairwise identity  
207 ( $I$ ; Belkhir et al. 2002). The probability that each sample was a random draw from a panmictic  
208 population was estimated in IDENTIX (Belkhir et al. 2002) by permutating across genotypes and  
209 generating 1000 randomized pseudo-samples under the assumption of no relatedness. The  
210 proportion of pseudo-samples that produced a larger mean pairwise identity than the observed  
211 mean pairwise identity was used to calculate the p-value for the null hypothesis that the sample  
212 represented a random draw from a panmictic population.

### 213 *Population structure*

214       We used contingency tests of allele frequency homogeneity to test the null hypothesis of  
215 panmixia between any pair of sample collections. Contingency tests were conducted using the  
216 methods of Raymond and Rousset (1995) as implemented in GENEPOP 4.0.7. For each locus,  
217 we estimated the exact probability that the observed allele frequencies were drawn from the same  
218 population using the Markov chain Monte Carlo (MCMC) methods to provide an unbiased  
219 estimate of the exact probability for each randomization test, we ran 100 batches of 5000  
220 replicates each, with 10 000 dememorisation steps. For each comparison we used the Fisher's

221 combined probability test ( $\chi^2_F$ ) as a composite test over all loci and used the false discovery rate  
222 (FDR) method of Narum (2006) to set a experiment-wise  $\alpha$  at the 0.05 level that accounts for  
223 multiple tests.

224 The level of genetic variation among populations was estimated by  $F_{ST}$  using the  $\theta$   
225 statistic of Weir and Cockerham (1984). Pairwise  $F_{ST}$  values were estimated in GENEPOP 4.0.7.  
226 Estimates of  $F_{ST}$  over all sample locations and associated 95% confidence intervals, generated by  
227 bootstrap sampling over loci, were calculated in FSTAT 2.9.3.2 (Goudet 2001). Testing for  
228 different ancestral histories between populations was accomplished by comparing global  
229 variance in allelic identity (i.e.,  $F_{ST}$ ) and allelic size (i.e.,  $R_{ST}$ ) measures of genetic difference.  
230 Under the null hypothesis ( $R_{ST} = F_{ST}$ ) differentiation is caused mainly by drift while under the  
231 alternative hypothesis ( $R_{ST} > F_{ST}$ ) stepwise mutations have contributed to differentiation (Hardy  
232 et al. 2003). SPAGEDI v1.2 (Hardy and Vekemans 2002) was used to calculate  $R_{ST}$  and to test  
233 for significant differences between  $F_{ST}$  and  $R_{ST}$  using the allele size permutation test of Hardy et  
234 al. (2003) ( $\alpha = 0.05$  with FDR adjustment). We used standard error estimates from SPAGEDI  
235 v1.2 to calculate 95% confidence intervals for the average pairwise  $R_{ST}$  estimates over all  
236 populations. To test for geographic structure and consistency with a stepping-stone model of  
237 gene flow among native populations with presumed historical connectivity we examined the  
238 correlation between the natural log of pairwise fluvial distances (km) and genetic differentiation  
239 using Mantel tests implemented in GENEPOP 3.7 (Rousset 1997).

240 Genetic relationships among populations were inferred by generating an unrooted  
241 neighbor-joining tree based on Cavalli-Sforza and Edwards (1967) chord distance. Confidence  
242 in the observed topology of the NJ-population tree was assessed using the bootstrap procedure in

243 PHYLIP v3.6 (Felsenstein 1992) based on 1000 resamples across loci. The consensus NJ-  
244 population tree was generated in PHYLIP v3.6.

245 *Effective population size*

246 We estimated the long-term effective population size ( $N_e$ ) using the heterozygosity-based  
247 methods of Ohta and Kimura (1973) and Hartl and Clark (1989). Both methods assume selective  
248 neutrality and predict that at mutation-drift equilibrium,  $N_e$  is a function of  $H_E$ . Ohta and Kimura  
249 (1973) assume a step-wise mutation model (SMM), whereas Hartl and Clark (1989) assume an  
250 infinite allele mutation model (IAM). We used the most commonly applied microsatellite  
251 mutation rate for fishes,  $\mu = 5 \times 10^{-4}$  (Estoup and Angers 1998).

252

253 (1) 
$$N_{eSMM} = \frac{\left[ \left( \frac{1}{1-H_E} \right)^2 - 1 \right]}{8\mu}$$
 (Ohta and Kimura 1973)

254

255 (2) 
$$N_{eIAM} = \frac{H_E}{4\mu(1-H_E)}$$
 (Hartl and Clark 1989)

256

257 The true long-term  $N_e$  should be in between  $N_{eSMM}$  and  $N_{eIAM}$  because these two models represent  
258 the extremes of the mutation process for microsatellite loci (Busch et al. 2007).

259 We estimated contemporary  $N_e$  of each population using the single-sample linkage  
260 disequilibrium method of Waples (2006) implemented in program LDNe (Waples and Do 2008).  
261 This method provides an estimate of the effective number of breeding adults that parented the  
262 sampled population. In the analysis we excluded all alleles with frequencies less than 0.02, and  
263 used the jackknife procedure to estimate the 95% confidence intervals.

264 *Tests for recent population declines*

265 Heterozygosity excess (Cornuet and Luikart 1996) and low ratios of allelic number to  
266 allelic size range (*M*-ratio; Garza and Williamson 2001) were used to screen populations for  
267 genetic signatures of recent bottlenecks. The two methods are expected to detect bottlenecks on  
268 different timescales allowing us to infer the time period that population decline or recovery  
269 occurred (Williamson-Natesan 2005; Spear et al. 2006). The *M*-ratio method remains sensitive  
270 to size reductions even up to 500 generations following the event while the heterozygosity-based  
271 methods is most powerful at detecting more recent bottlenecks (e.g., 0.2 – 4.0  $N_e$  generations;  
272 Luikart and Cornuet 1998). We expected that: (a) native Missouri River populations (1-9) would  
273 exhibit bottlenecks detectible by both methods because of major habitat alterations 50-100 yr ago  
274 and subsequent demographic declines; (b) derived conservation populations (10-12) would show  
275 more recent bottlenecks if hatchery effects were significant; and (c) introduced populations (13-  
276 17) founded > 50 years ago may exhibit founder effects (significant *M*-ratio).

277 Tests for heterozygosity excess were done with the program BOTTLENECK v1.2.02  
278 (Piry et al. 1999) using a two-phase model (TPM) of microsatellite mutation (Di Rienzo et al.  
279 1994) with parameter settings of 95% SMM, 5% IAM and 12% variance in multi-step mutations  
280 (i.e., presumed model for microsatellites: Piry et al. 1999; Lippé et al. 2006). Significance of  
281 heterozygosity excess observed in a population was evaluated by a one-sided Wilcoxon signed  
282 rank test ( $\alpha=0.05$ ) comparing the level of deviation from the null hypothesis of 50:50  
283 heterozygosity deficiency:excess based on 5000 simulation iterations. *M*-ratios were calculated  
284 in M\_P\_VAL (Garza and Williamson 2001). The *M*-ratio is a measure of the number of alleles  
285 (*k*) to the allele size range (*r*). The *M*-ratio (*k/r*) is expected to be small in a recently-  
286 bottlenecked population because *k* is reduced faster than *r*. Program M\_P\_VAL implemented a

287 model of microsatellite evolution assuming 88% one-step mutations ( $p_g$ ), and 2.8 average size of  
288 non one-step mutations ( $\Delta_g$ ) (Garza and Williamson 2001). We explored a range of sizes for the  
289 pre-bottlenecked population ( $N_e = 500$  and 5000) and a microsatellite mutation rate of  $\mu = 5 \times$   
290  $10^{-4}$  to estimate  $\theta (= 4 \times N_e \times \mu)$ . Statistical significance of the observed  $M$ -ratio was calculated  
291 using the program *Critical\_M* (Garza and Williamson 2001), with the critical value ( $M_c$ ) for each  
292 sample such that 95% of 10 000 simulations of an equilibrium population had  $M$ -ratio  $> M_c$ .

## 293 **Results**

### 294 **Within population diversity**

295 We observed moderate to high levels of genetic diversity in Arctic grayling across all 10  
296 microsatellite loci. Expected heterozygosity ( $H_E$ ) over all loci ranged from 0.63 in Sunnyslope  
297 Canal to 0.89 in four Big Hole River samples (Table 3; see also Supplement 1), and averaged  
298 0.80 across all samples. The number of alleles per locus ( $N_A$ ) ranged from 5.0 in Sunnyslope  
299 Canal to 16.7 in Swamp Creek (a tributary to the Big Hole River), and averaged 12.7 across all  
300 samples. The Big Hole River group exhibited higher allelic richness using a sample of 36 genes  
301 ( $N_A^{36} = 13.24$ ) and  $H_E$  (0.89) than the other native populations combined (i.e., populations #6-9;  
302  $N_A^{36} = 9.46$  and  $H_E = 0.79$ ,  $p < 0.05$  for both comparisons).

Table 3  
near here

303 Genotypic frequencies were in HWE for 175 of 180 tests. Only Axolotol Lake (11),  
304 Miner Lake (7), and Bozeman Fish Technology Center (12) were out of HWE for 3 loci, 1 locus,  
305 and 1 locus, respectively (Table 3). Significant linkage disequilibrium was observed at only 10  
306 of 801 pairs of loci tested over all populations, and all but one of linked pairs occurred in Axolotl  
307 Lake ( $n = 9$  pairs of loci).

308 Individuals within sample populations did not appear to be highly related to one another,  
309 with a few exceptions. None of the populations violated the test assumption of being more

310 related than expected based on a random draw individuals from a panmictic population (lowest  
311 one-sided p-value = 0.053, average p-value = 0.56, SD = 0.32). Most populations had  
312 relatedness coefficients ( $I$ ) less than 0.25 (i.e., analogous to half siblings; Table 3). Fish sampled  
313 in the Big Hole River were less related on average (mean  $I = 0.14$ ) than fish sampled from the  
314 other native Missouri River populations (mean  $I = 0.25$ ). The Sunnyslope Canal was nearly  
315 consistent with a population of full siblings ( $I = 0.49$ ).

### 316 **Population structure**

317 We observed substantial genetic subdivision among grayling populations outside of the  
318 connected Big Hole River (Table 4). Pairwise  $F_{ST}$  estimates generally mirrored the differences  
319 in allele frequencies: genetic differentiation among demes within the connected Big Hole River  
320 was minimal ( $F_{ST} \leq 0.0055$ , homogeneity in allele frequencies in 8 of 10 comparisons) whereas  
321 differentiation between the Big Hole River group (i.e., #1-5) and all other wild native and  
322 naturalized populations was greater ( $F_{ST} \geq 0.035$ , heterogeneity in allele frequencies in 10 of 10  
323 cases). The greatest differentiation was generally observed between Canadian-origin populations  
324 (Fuse Lake, Fond du Lac) and those from the Missouri River system (range  $F_{ST} = 0.13-0.31$ ).  
325 Canadian-origin populations contained many alleles not found in any of the Montana  
326 populations, a high frequency of private alleles at six loci, and a very different distribution of  
327 allele size ranges (Supplement 2). Pairwise  $F_{ST}$  values between the five native grayling  
328 populations in Montana (#1-5, 6, 7, 8, and 9) ranged from at least 0.071 between the combined  
329 Big Hole River group (#1-5) and all other populations, to 0.174 between the Red Rock lakes (#8)  
330 and the Madison River-Ennis Reservoir (#9).

331 The global  $R_{ST}$  of 0.25 (95% CI 0.17-0.33;  $n = 14$  populations with #1-5 group and #6-  
332 18) was much larger than the corresponding global  $F_{ST}$  of 0.10 (95% CI 0.07-0.15) indicating

Table 4  
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333 that  $F_{ST}$  underestimates the actual levels of genetic structure in some comparisons. The allele  
334 size permutation test identified 41 of 91 pair-wise comparisons of populations in which  $R_{ST} >$   
335  $F_{ST}$  (Table 4; the 91 comparisons exclude individual estimates for sample sites within the  
336 connected Big Hole River #1, 2, 3, 4, and 5). Pairwise  $R_{ST}$  values were largest between  
337 Canadian-origin populations (#14, 18) and the upper Missouri River populations (#1-9), with the  
338 largest value ( $R_{ST} = 0.76$ ) observed between Mussingbrod Lake (#6) and Fond du Lac (#18).  
339 Mussingbrod Lake (#6) was the most genetically distinct among the native Missouri River  
340 populations (# 1-9) based on pairwise  $R_{ST}$  estimates. The introduced population in Bobcat Lake  
341 (#16) had  $R_{ST} > F_{ST}$  for all comparisons except for with Red Rock lakes (#8).

342 The neighbor-joining dendrogram revealed five genetically distinct groups (Figure 2).  
343 Distinctiveness of the Canadian-origin populations was highly supported with a 100% bootstrap  
344 support for the separation of these two populations from all others. The Big Hole River group  
345 was also very distinct with a 96% bootstrap support. Three moderately supported groups  
346 consisting of Red Rock Creek, Madison River, and the two isolated lakes in the Big Hole River  
347 watershed were also identified, although the relationships of these three groups to one another  
348 was not well defined. Affinity of the naturalized populations with their sources was apparent in  
349 the topology of dendrogram. Overall levels of genetic differentiation were greater among natural  
350 populations than among naturalized populations and their source populations.

351 Clusters of individuals from native grayling locations in the Missouri River (# 1-9)  
352 identified using the Bayesian method of STRUCTURE (Pritchard et al. 2000) were consistent  
353 with patterns of population structure observed with genic tests,  $F_{ST}$ ,  $R_{ST}$  and the phylogenetic  
354 analysis (Supplement 3). Evaluation of the assignment of individuals to six clusters had a clear  
355 biological interpretation based on geography and physical isolation. There was little support for

Figure 2  
near here

356 population structure within the connected Big Hole River (#1-5), however fish from this system  
357 did form a cluster that was distinct from the other locations sampled. Fish collected from  
358 Mussingbrod Lake (#6), Miner Lake, Red Rock Lakes (#7), Madison River (#8), and Fuse Lake  
359 (#14) all formed distinct genetic clusters.

360 We did not detect a statistically significant isolation-by-distance effect based on based on  
361 genetic differentiation that considered mutation ( $R_{ST}$ ,  $n = 10$ , Spearman's  $r_s = 0.164$ , Mantel  $p$ -  
362 value = 0.35) among the five presumed native populations in the upper Missouri River system  
363 (Supplement 4). Differentiation based on genetic drift ( $F_{ST}$ ) did indicate isolation-by-distance  
364 (Supplement 4), however  $F_{ST}$  underestimates differentiation among sample pairs that include  
365 Miner and Mussigbrod lakes where differences are due, in part, to mutation (Table 4).

#### 366 **Effective population size**

367 Historical  $N_e$  of native Missouri River grayling ranged from ~1800 to 20 000, and was  
368 highest for the Big Hole River group (~4000 – 20 000) and derived populations (Table 5).  
369 Estimates of contemporary  $N_e$  were generally much lower (11 of 15 populations < 500), though  
370 precision was low in some cases and six populations had estimates or upper confidence limits  
371 that were infinite. Contemporary  $N_e$  for most native Missouri River populations were typically  
372 between 150 and 300 (range 162.3-286.3), with the exception of Mussigbrod Lake ( $N_e = 1496.8$ ).  
373 Effective size for the Big Hole River group was 207.6, but  $N_e$  for two derived populations  
374 (Axolotl Lake, Bozeman FTC) was much less (29.5 and 38, respectively).

375 Six populations stood out in a bivariate plot of the mean number of alleles per locus  
376 versus the mean number of private alleles per locus (Figure 3). Sunnyslope Canal and Madison  
377 River both had low number of alleles and a low level of private alleles suggesting these  
378 populations have a small  $N_e$  relative to the rest of the populations and they are genetically similar

Table 5  
near here

Figure 3  
near here

379 to the other Montana populations. The Canadian-origin populations had the highest levels of  
380 private alleles and an intermediate level of mean number of alleles; indicating long-term genetic  
381 isolation relative to Montana populations. Among Montana populations, Miner Lake had the  
382 most private alleles per locus suggesting it has had a relatively low level of gene flow with the  
383 other native Montana populations. The highest number of alleles was observed in the Big Hole  
384 River group which is consistent with this population having a comparatively large  $N_e$ .

### 385 **Population bottlenecks**

386 The Ruby River and Axolotl Lake samples experienced recent bottlenecks based on the  
387 heterozygosity excess test. Thirteen of 14 sample populations exhibited bottlenecks by the  $M$ -  
388 ratio test using least one of the presumed  $\theta$  values. Only the Big Hole River group did not,  
389 though its  $M$ -ratio estimate (0.818) was close to the critical value at  $\theta = 1$  ( $M_c = 0.813$ ).

## 390 **Discussion**

### 391 **Ancestry and population structure in Missouri River Arctic grayling**

392 Microsatellite DNA markers confirm earlier findings that Arctic grayling from the upper  
393 Missouri River have been separated from Canadian populations for a long time, perhaps even  
394 before the most recent glacial advance (Redenbach and Taylor 1999). Private alleles in the  
395 Canadian populations were at high frequency with size distributions distinct from Montana  
396 samples, and very high  $R_{ST}$  values indicate that mutation, in addition to genetic drift, is  
397 responsible for the differentiation.

398 Arctic grayling from the connected Big Hole River form a genetic group distinct from  
399 other native populations in the Madison River-Ennis Reservoir and Red Rock lakes. In turn,  
400 there is also moderate support that these latter two populations each form discrete genetic  
401 groups. Population groupings based on protein electrophoresis previously found no genetic

402 distinction between Big Hole and Madison river grayling (Everett 1986; R. Leary, University of  
403 Montana, unpublished report, 1990), perhaps because only 2 of 39 allozyme loci were  
404 polymorphic for Montana samples.

405         With the exception of the Canadian-origin Fuse Lake population, naturalized lacustrine  
406 Arctic grayling populations in the dataset appear to trace their ancestry to adfluvial grayling from  
407 Red Rocks lakes. This differs from the historical interpretation that fish from the Madison River  
408 made a significant genetic contribution to naturalized populations derived from the Georgetown  
409 Lake and Washoe Park Hatchery populations (Kelley 1931; Everett 1986; Kaya 1990). These  
410 now-extinct stocks were a major source of further grayling introductions in Montana (Kaya  
411 2000; MFWP 2005b). We were not able to locate archived tissue from Georgetown and  
412 Washoe grayling so the genetic composition of the stocked fish is unknown, but the most direct  
413 explanation for genetic similarity among naturalized populations is a common origin. We  
414 hypothesize a founder effect from stocking of Red Rock-origin fish is the reason for high  $R_{ST}$  in  
415 Bobcat Lake relative to all other populations except for Red Rock lakes. The distribution of  
416 allele sizes in Bobcat Lake grayling was consistent with other Missouri River samples, and  
417 Bobcat Lake grayling also cluster with Missouri River populations (and not those from Canada).

418         Genetic and biogeographic data suggest that Arctic grayling in Miner and Mussigbrod  
419 lakes may be remnant native populations. The level of genetic differentiation in these  
420 populations also indicates they have been demographically independent for some time.  
421 Mussingbrod Lake had  $R_{ST} > F_{ST}$  relative to the connected Big Hole River and Miner Lake,  
422 indicating it has been isolated long enough for mutation to have contributed to its genetic  
423 distinction. In contrast, Miner Lake had  $R_{ST} = F_{ST}$  relative to the connected Big Hole River,  
424 indicating genetic drift has caused the observed differentiation and the population has been

425 isolated for less time than the Mussingbrod Lake population. Stocking of hatchery fish could  
426 also account for large  $R_{ST}$  values in these lakes, however, both Miner and Mussigbrod lakes were  
427 strongly differentiated from the Red Rock lakes population with  $R_{ST} > F_{ST}$  for both comparisons  
428 ( $F_{ST} = 0.15-0.16$ ;  $R_{ST} = 0.32-0.48$ ). In addition, Miner and Mussigbrod lakes did not cluster with  
429 other naturalized populations apparently derived from Red Rock lakes. Such differentiation  
430 would be unlikely if Red Rocks grayling were stocked in those waters and made a significant  
431 genetic contribution to the extant population. Presence of other native fishes in high-elevation  
432 lakes in the upper Big Hole River watershed (e.g., Vincent 1963) demonstrates historical  
433 connectivity with the main-stem river. Indigenous burbot (*Lota lota*) and longnose sucker  
434 (*Catostomus catostomus*) populations in Miner and Mussigbrod lakes (B. Snyder, MFWP,  
435 personal communication, 2006) implies natural colonization by grayling was also possible.  
436 Consequently, we conclude that grayling populations in Miner and Mussigbrod lakes are not  
437 solely derived from stocking.

438         We found no evidence for introgression of Canadian-origin fish in Missouri River  
439 grayling samples. Moreover, we did not find any indication that extant native Arctic grayling in  
440 the Big Hole River, Madison River-Ennis Reservoir, or Red Rock lakes interbred with stocked  
441 fish originating from within the Missouri River basin or that these native populations now  
442 represent genetic admixtures. Genetic homogenization could be one consequence of  
443 interbreeding with hatchery grayling derived from a single source (e.g., Red Rock lakes). In  
444 contrast, all the native Missouri River populations appear to form distinct genetic groups.  
445 Everett (1986) also concluded that stocking was not successful or contributed little to the  
446 spawning population in the Big Hole River, and that geographic structuring was the most direct  
447 explanation for genetic (allozyme) differences among grayling in Montana and Wyoming.

448 *Thymallus* populations influenced by intraspecific stocking sometimes exhibit greater  
449 genetic diversity than unaltered native populations (e.g., Gum et al. 2003). The Big Hole group  
450 was the most diverse sample population, but to attribute this to admixture from stocking, one  
451 would have to discount landscape genetic arguments that greater diversity in the Big Hole  
452 population (relative to other native populations) could be a function of multiple spawning  
453 locations, and greater habitat extent (e.g., Neville et al. 2006) or habitat connectivity (e.g.,  
454 Costello et al. 2003; Taylor et al. 2003; Wofford et al. 2005). A direct test of introgression (e.g.,  
455 Koskinen et al. 2002b; Gum et al. 2006) was not possible for indigenous Missouri River grayling  
456 because we did not have any samples collected before stocking. Theoretically, stocking from  
457 different sources, or stocking from a single source following by genetic drift, could have affected  
458 the genetic characteristics of Big Hole River grayling. This appears unlikely given our data and  
459 considering that most attempts to introduce Arctic grayling into riverine habitats generally have  
460 not been successful (Northcote 1995; Kaya 2000; MFWP 2004).

461 Little or no recent gene flow has occurred among grayling populations in the Big Hole,  
462 Madison, and Beaverhead river sub-basins; or between Mussigbrod and Miner lakes in the Big  
463 Hole River watershed. In contrast, the data suggest recent genetic exchange among spawning  
464 sites within the connected Big Hole River, as the demes sampled were largely panmictic. The  
465 spatial scale of population structuring for Missouri River grayling (over 100s km) is similar to  
466 observation of Arctic grayling from rivers in Alaska (Hop and Gharrett 1989) and British  
467 Columbia (Stamford and Taylor 2005). Landscape genetic variation in stream salmonids is  
468 driven by historical and contemporary factors such as glaciation and habitat modification (e.g.,  
469 Costello et al. 2003; Taylor et al. 2003; Stamford and Taylor 2005); species-specific differences  
470 in behavior and life history (e.g., Wenburg et al. 1998; Heggenes et al. 2006; Neville et al. 2006),

471 and ecological context (e.g., Haugen and Vøllestad 2000; Koskinen et al. 2001). Population  
472 genetic structure in *Thymallus* has been more frequently detected among sub-basins (Gum et al.  
473 2003; Koskinen et al. 2002a; but see Koskinen et al. 2001) relative to other salmonids that show  
474 differentiation at finer scales, such as among or within tributary streams (e.g., Carlsson et al.  
475 1999; Taylor et al. 2003; Young et al. 2004).

476 We infer the contemporary landscape genetic pattern observed in native Missouri River  
477 grayling represents historical population structure, but recognize dams may have fostered some  
478 of the observed differentiation among Big Hole, Madison, and Red Rock population. Restricted  
479 gene flow promotes genetic drift (Waples 1998), and habitat fragmentation can strongly  
480 influence genetic differentiation and diversity in stream fishes (Costello et al. 2003; Wofford et  
481 al. 2005), especially those like Arctic grayling (e.g., Stamford and Taylor 2005) that can move  
482 hundreds of km among complementary habitats (Armstrong 1986; Northcote 1995; MFWP  
483 2003). Analysis of museum specimens may help determine whether contemporary factors have  
484 altered the genetic architecture that existed among Missouri River grayling populations prior to  
485 Euro-American settlement.

#### 486 **Demographic status and population declines inferred from genetic data**

487 Arctic grayling in the Missouri River system were historically very abundant, with long-  
488 term  $N_e$  ranging from a few thousand to tens of thousands. There is some uncertainty as to the  
489 timeframe and demographic scale associated with long-term estimates of effective population  
490 size (Waples 1991), but most authors agree that these estimates can be interpreted as the  
491 harmonic mean of the  $N_e$  in each generation among populations within a geographic region  
492 (Kalinowski and Waples 2002). In this case, the long-term estimates are best interpreted as  $N_e$   
493 for Arctic grayling populations in the Missouri basin since the Wisconsin glacier receded

494 approximately 10 000 years ago. Contemporary  $N_e$  for individual populations was significantly  
495 less than the long-term  $N_e$ . Most populations did not exhibit inbreeding (i.e., contemporary  $N_e >$   
496 50), but  $N_e$  were low enough to raise concern about maintaining adaptive potential.

497 Most populations showed genetic signatures of recent population declines, but the  
498 approximate timing of these bottlenecks varied. This study was correlative so we cannot directly  
499 attribute a bottleneck to a specific causal factor, but the observations were generally consistent  
500 with the demographic histories of some populations. The most recent bottlenecks  
501 (heterozygosity excess) were detected in Axotl Lake and Ruby River grayling, so we presume  
502 that 20 yr of captivity and hatchery culture are proximate cause. Bottlenecks from declines  
503 occurring two or more decades ago ( $M$ -ratio, e.g., Spear et al. 2006) were detected in every  
504 sample except the Big Hole River group.

505 We expected to find a bottleneck in the Big Hole given the population is at low  
506 abundance. Perhaps the genetic signatures of a bottleneck are not apparent despite a  
507 demographic decline (e.g., see Busch et al. 2007), or the decline has been more gradual or  
508 smaller compared with other native populations. Arctic grayling still spawn in multiple  
509 locations in the Big Hole River and the main-stem migratory corridor is largely intact. In  
510 contrast, fragmentation of main-stem habitats likely contributed to bottlenecks observed in Red  
511 Rock lakes and the Madison River. In the Red Rock River system grayling were extirpated from  
512 numerous tributary streams during the last 100 yr, and the remaining adfluvial population in the  
513 headwater lakes was isolated by a barrier installed in 1957 (Nelson 1954; Vincent 1962; Mogen  
514 1996). In the Madison River, the construction of impassible Ennis Dam >100 yr ago  
515 constrained, if not eliminated, any expression of the fluvial life history for grayling (Vincent  
516 1962; Kaya 1992). The residual (adfluvial) Madison River population may have been further

517 affected by draw downs of Ennis Reservoir in the 1960s-1980s which reduced surface area by up  
518 to 15-50% (MFWP 1990).

519 The Miner and Mussigbrod lake grayling populations have not been extensively  
520 monitored, so the causes of abrupt population decline are unknown. Loss of connection to the  
521 main-stem Big Hole River, winter oxygen deficits (die offs), or competition with stocked  
522 grayling are possible factors. Few demographic data are available for naturalized lake  
523 populations, but the timing of bottlenecks suggests possible founder effects. The genetically  
524 depauperate and inbred Sunnyslope Canal population has been affected by irrigation  
525 management and seasonal dewatering (Barndt and Kaya 2000)

#### 526 **Conservation implications**

527 First, the multiple demes within the Big Hole River should tentatively be considered a  
528 single management unit, and all native grayling populations in the Missouri River are  
529 demographically independent from one another. Genetic approaches to defining conservation  
530 and management units have been proposed (e.g., Crandall et al. 2000; Palsbøll et al. 2007), but  
531 the presence of main-stem migration barriers currently dictates that these native populations be  
532 managed as separate units. Conservation efforts should immediately focus on reducing  
533 demographic risks by increasing the local abundance and distribution of the individual  
534 populations, especially in the Big Hole River, Madison River, and Red Rock lakes. We presume  
535 these populations were historically linked by occasional gene flow and must have exhibited some  
536 metapopulation-like structure (e.g., Rieman and Dunham 2000). In the future, facilitating habitat  
537 connectivity among sub-basins occupied by native populations may help reduce genetic and  
538 stochastic risks. The challenge in doing this, however, will be considerable given existing

539 fragmentation and habitat alteration, and possible threats from further nonnative trout invasions  
540 (Fausch et al. In Press).

541         Second, the Big Hole River population represents an important genetic reservoir for the  
542 species within the Missouri River basin, and is also the last example of the riverine, migratory  
543 (fluvial) ecotype (Kaya 1992). Genetic and life history diversity should help buffer populations  
544 against environmental variation, catastrophe, and anthropogenic stressors (e.g., Rieman and  
545 McIntyre 1993; Crandall et al. 2000; Fausch et al. 2006). By these criteria, the Big Hole River  
546 should be a high priority for protection and restoration. Current management focuses on  
547 implementation of a conservation agreement in the upper Big Hole River (MFWP 2006).

548         Third, efforts to use captive populations to wild re-establish fluvial populations in  
549 indigenous waters will probably continue to challenge grayling restoration biologists. A slight  
550 reduction in genetic diversity has occurred in the ancestors of the captive population derived  
551 from Big Hole River grayling, and the Axolotl Lake and Ruby River samples also showed  
552 genetic signals of a recent bottleneck. There are no data to indicate an effect on translocation  
553 success, but genetic effects of captivity and culture may be unavoidable (e.g., Frankham 2008).

554         Drought, nonnative trout, and a lack of imprinting by stocked fish may have limited  
555 reintroduction success of fluvial Arctic grayling in Montana (MFWP 2003, 2007). A general  
556 lack of connected, main-stem river habitats accessible to grayling may further constrain  
557 expression of a migratory life history at translocation sites. Alternatively, local adaptation may  
558 also be important. Divergence among populations measured by molecular markers can  
559 potentially indicate differences in behavior, ecology, life history, and physiology (Stamford and  
560 Taylor 2005) that influence survival in specific habitats (Merilä and Crnokrak 2001; e.g., in  
561 *Thymallus*, see Koskinen et al. 2001; Salonen 2005). If genetic differentiation in the Big Hole

562 River group is concordant with local adaptation (Taylor 1991), then a lack of ecological  
563 exchangeability (*sensu* Crandall et al. 2000) between sub-basins may in part explain why  
564 translocations using Big Hole River-derived grayling have yet to establish a self-sustaining  
565 population in another river. Common-garden experiments that test for unique or population-  
566 specific traits might provide insight into local adaptation and aid future translocations.

567         Local adaptation in Big Hole River Arctic grayling would seemingly present a  
568 conundrum for future reintroduction efforts using that stock, however few alternatives exist.  
569 Adfluvial Arctic grayling have not been successfully transplanted in to rivers in Montana (Kaya  
570 1992), perhaps because of heritable behavioral differences between adfluvial and fluvial stocks  
571 (e.g., Kaya 1991; Kaya and Jeanes 1995). Madison River grayling are extremely rare (MFWP  
572 2007), no brood reserve exists for the population, and the contemporary  $N_e$  (162.3) is based  
573 mostly on fish captured 1-2 generations ago. Moreover these fish now exhibit an adfluvial life  
574 history, and it is unclear whether their descendents would re-express a fluvial life history. In the  
575 Missouri River basin, Big Hole River grayling theoretically provide a broader template for  
576 adaptation to novel environments or future environmental challenges because of their greater  
577 genetic diversity (Franklin and Frankham 1998, Lynch and Lande 1998). On the other hand,  
578 genetically depauperate populations can persist (e.g., Sunnyslope Canal). If maintaining  
579 diversity in captive populations (of Big Hole grayling) is important, then supplementation from  
580 the wild population will be needed to limit genetic erosion. Adult grayling are presently at low  
581 abundance in the Big Hole River (MFWP 2005a, 2007), so the population may not be able to  
582 demographically support any removal of gametes. Translocations may have to proceed using the  
583 genetic resources already in captivity; with supplementation of captive or reintroduced  
584 populations occurring after the Big Hole population experiences a demographic recovery.

585 **Acknowledgements**

586 The authors dedicate this manuscript to the memory of Rebecca J. Everett. D. Campton  
587 (USFWS) provided a thoughtful literature review and synthesis that framed many of the  
588 questions addressed in this study. R. Leary (University of Montana and MFWP) provided a  
589 helpful review of the original study proposal. M. Diggs completed the study's lab work. Erik  
590 Olsen provided database support. USFWS Region 6 and the US Bureau of Reclamation  
591 provided funding. M. Wilson (USFWS) provided administrative guidance during project  
592 conception. US Forest Service, MFWP, and numerous volunteers helped collect Arctic grayling  
593 finclips in Montana. A. Steed (Montana State University) contributed samples from Grebe Lake,  
594 Yellowstone National Park; and J. Merkowsky provided samples from northeastern  
595 Saskatchewan, Canada. The findings and conclusions in the article are those of the authors and  
596 do not necessarily represent the views of the U.S. Fish and Wildlife Service.

597

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**Table 1.** Presumed origin, location, sample size (N), and sample collection years for 18 native and introduced Arctic grayling (*Thymallus arcticus*) populations in the upper Missouri River and one from the Mackenzie River system, Canada.

No.	Sample population	Latitude (°N)	Longitude (°W)	Elevation (m)	N	Years
Native – upper Missouri River basin						
Connected Big Hole River						
1	Steel Creek <sup>a</sup>	45.66682	-113.46601	1826	27	2006
2	Swamp Creek <sup>a</sup>	45.64978	-113.44271	1823	38	2005, 2006
3	Big Hole River near Wisdom, MT <sup>a</sup>	45.61755	-113.45758	1844	22	2006
4	Fishtrap Creek <sup>a</sup>	45.8697	-113.22847	1779	28	2005, 2006
5	Lamarche Creek <sup>a</sup>	45.8834	-113.20211	1780	29	2005, 2006
Upper Big Hole River watershed						
6	Mussigbrod Lake <sup>b</sup>	45.79078	-113.61149	1979	48	2006
7	Miner Lake <sup>b</sup>	45.3248	-113.56747	2125	37	2006
Red Rock lakes, Beaverhead River						

8	Red Rock Creek	44.61359	-111.68807	2016	48	2005
	Madison River					
9	Madison River-Ennis Reservoir	45.40931	-111.69449	1469	27	1996 (n=23), 1999 (1), 2004 (1), 2006 (2)
	Captive reserve or conservation – derived from Big Hole River					
10	Ruby River	45.00863	-111.96207	1880	48	2006
11	Axolotl Lake	45.2258	-111.8713	2229	55	2006
12	Bozeman Fish Technology Center (BFTC)	45.70802	-110.9774	1497	48	2005
	Introduced – other sources (source)					
13	Odell Lake (unknown)	45.57523	-113.23231	2543	46	2006
14	Fuse Lake (Canada)	46.24785	-113.72297	2347	47	2006
15	Grebe Lake (upper Missouri River)	44.75083	-110.55778	2447	47	2002, 2005
16	Bobcat Lake (unknown)	45.6219	-113.2206	2569	47	2006
17	Sunnyslope Canal (unknown)	47.60490	-112.09710	1293	50	2006
	Native population – Saskatchewan, Canada					
18	Fond du Lac, Athabaska River system	59.24341	-106.41357	202	34	1987

<sup>a</sup> Putative demes for fluvial Arctic grayling within the Big Hole River and connected tributaries.

<sup>b</sup> Putative remnant populations isolated by habitat degradation and water development.

**Table 2.** Documented stocking of hatchery-cultured Arctic grayling (*Thymallus arcticus*) into waters containing indigenous or presumed indigenous populations.

	Population	Years		Total number stocked	Hatchery source
		stocked	stocked		
1-5	Big Hole River (group)	1937-1957	14 732 193 <sup>a</sup>	Washoe Park Hatchery (MFWP 2005b) <sup>b</sup>	
6	Mussigbrod Lake	1934-1955	5 951 847	Washoe Park Hatchery (MFWP 2005b) <sup>b</sup>	
7	Miner Lake	1933-1952	3 220 776	Washoe Park Hatchery (MFWP 2005b) <sup>b</sup>	
8	Red Rock	1899-1938,	Unknown,	Bozeman National Fish Hatchery (Randall 1978),	
		1946-1962	103 704	Ennis National Fish Hatchery (Randall 1978, MFWP 2005b)	
9	Madison River- Ennis Reservoir	1928-1961	2 690 725 <sup>c</sup>	Washoe Park during 1928-1934, Ennis National Fish Hatchery during 1946-1962 (MFWP 2005b)	

<sup>a</sup> Number includes records for Arctic grayling planted into the Big Hole River and its tributaries. Number excludes 1080 Arctic grayling from Somers Hatchery (also known as Flathead Lake Salmon Hatchery) planted in 1962, and 622 Arctic grayling from the Bozeman Fish Technology Center planted in 1992 and 1993.

<sup>b</sup> The Washoe Park Hatchery is operated by the state of Montana, and was historically referred to as the “Anaconda Hatchery”.

<sup>c</sup> Numbers for the Madison River includes records for Arctic grayling planted into Odell Creek; Ennis Reservoir; the lower, middle, and upper segments of the Madison River; and the South Fork Madison River (MFWP 2005b).

**Table 3.** Population genetic characteristics for 18 Arctic grayling (*Thymallus arcticus*) sample populations across 10 microsatellite loci.

Sample population	N	N <sub>A</sub>	N <sub>A</sub> <sup>36</sup>	N <sub>P</sub>	H <sub>O</sub>	H <sub>E</sub>	H <sub>WE</sub>	LD	Relatedness (I)
Native – upper Missouri River basin									
Connected Big Hole River									
1 Steel Creel	27	14.5	12.9	-	0.89	0.88	0	0	0.1569
2 Swamp Creek	38	16.7	13.5	-	0.87	0.89	0	0	0.1503
3 Big Hole River	22	14.7	13.7	-	0.86	0.89	0	0	0.1365
4 Fishtrap Creek	28	14.3	12.8	-	0.90	0.89	0	0	0.1480
5 Lamarche Creek	29	15.4	13.2	-	0.88	0.89	0	0	0.1532
1-5 Big Hole River (group) <sup>a</sup>	144	19.3	13.3 <sup>b</sup>	5	0.88	0.89	0	0	0.1438
Upper Big Hole River watershed									
6 Mussigbrod Lake	48	10.9	9.1	1	0.78	0.78	0	0	0.2437
7 Miner Lake	37	13.1	10.7	3	0.79	0.82	1	0	0.2101

Red Rock lakes, Beaverhead River										
8	Red Rock Creek	48	13.3	10.4	2	0.79	0.80	0	0	0.2181
Madison River										
9	Madison River-Ennis Reservoir	27	8.4	7.7	0	0.73	0.74	0	0	0.3285
Captive reserve and conservation – derived from Big Hole River										
10	Ruby River	48	14.3	11.7	0	0.90	0.88	0	0	0.1777
11	Axolotl Lake	55	15.0	11.8	0	0.87	0.88	4	9	0.1750
12	BFTC	48	13.7	10.8	1	0.85	0.85	1	1	0.2095
Introduced – other sources										
13	Odell Lake	46	14.0	11.0	2	0.84	0.83	0	0	0.1989
14	Fuse Lake	47	10.8	8.5	6	0.74	0.75	0	0	0.2675
15	Grebe Lake	47	13.7	10.6	2	0.84	0.85	0	0	0.2053
16	Bobcat Lake	47	9.9	8.3	3	0.78	0.80	0	0	0.2713
17	Sunnyslope Canal	50	5	4.5	0	0.62	0.63	0	0	0.4922
Native – Saskatchewan, Canada										
18	Fond du Lac	34	11.7	10.4	7	0.68	0.76	0	0	0.2753

**Note:**  $N$  = sample size,  $N_A$  = numbers of alleles,  $N_A^{36}$  = rarefaction measure of allelic richness using a sample of 36 genes,  $N_P$  = number of private alleles,  $H_O$  = observed heterozygosity,  $H_E$  = expected heterozygosity,  $HWE$  = number of loci not in Hardy-Weinberg equilibrium,  $LD$  = pairs of loci with significant linkage disequilibrium,  $I$  = mean pairwise relatedness estimates for multi-locus genotypes (after Belkhir et al. 2002) where values range from 0 for unrelated individuals to 0.5 for full siblings.

<sup>a</sup> The Big Hole River group (1-5) is the amalgamation of samples populations 1 through 5 (i.e., Steel Creek, Swamp Creek, Big Hole River near Wisdom, Fishtrap Creek, and Lamarche Creek).

<sup>b</sup> Table value for allelic richness estimated using HP-RARE 1.0 (see Methods). Allelic richness estimated using FSTAT 2.9.3.2 slightly lower ( $N_A^{36} = 13.24$ ; see Results, Within population diversity)

**Table 4.** Pairwise  $F_{ST}$  (below diagonal) and  $R_{ST}$  estimates (above diagonal) for Arctic grayling (*Thymallus arcticus*) at 10

microsatellite loci.

Population	Population number									
	1	2	3	4	5	1-5	6	7	8	9
1-Steel	-	-0.0096	-0.0083	-0.0047	-0.0063	NA	<b>0.3536</b>	0.0859	<b>0.1364</b>	0.0628
2-Swamp	0.0036 <sup>a</sup>	-	0.0004	-0.0031	-0.0106	NA	0.2905	0.0694	<b>0.1326</b>	0.0638
3-Wisdom	-0.0027 <sup>a</sup>	0.0019 <sup>a</sup>	-	-0.0125	-0.0054	NA	<b>0.381</b>	0.1167	0.0897	0.0547
4-Fishtrap	0.0052	0.0019 <sup>a</sup>	-0.0052 <sup>a</sup>	-	-0.0118	NA	0.3488	0.0995	0.0861	0.0509
5-Lamarche	0.0053	0.0055 <sup>a</sup>	-0.0019 <sup>a</sup>	-0.003 <sup>a</sup>	-	NA	<b>0.291</b>	0.0755	0.116	0.0586
1-5-BigHole (group)	NA	NA	NA	NA	NA	-	<b>0.2756</b>	0.086	0.1112	0.0506
6-Mussigbrod	0.0789	0.0805	0.0772	0.0743	0.0719	0.0719	-	<b>0.3075</b>	<b>0.4784</b>	0.4246
7-Miner	0.0789	0.072	0.072	0.0728	0.0777	0.072	0.1199	-	<b>0.3172</b>	0.1975
8-Red Rock	0.0808	0.0747	0.0699	0.0694	0.0782	0.0708	0.1604	0.1501	-	0.1442
9-Madison-Ennis	0.0725	0.0789	0.0806	0.076	0.0835	0.0726	0.1441	0.1496	0.1739	-
10-Ruby	0.023	0.0202	0.0128	0.0142	0.0191	0.0171	0.0937	0.075	0.0828	0.0987
11-Axolotl	0.0232	0.0155	0.0134	0.0138	0.0182	0.0162	0.0981	0.0758	0.0734	0.0938

12-BFTC	0.0318	0.0233	0.024	0.0237	0.0293	0.025	0.1044	0.086	0.0929	0.1023
13-Odell	0.0537	0.0579	0.0492	0.0464	0.0568	0.0508	0.1389	0.1329	0.0222	0.1246
14-Fuse	0.1375	0.1362	0.1399	0.1404	0.1414	0.1301	0.1962	0.1862	0.2021	0.1876
15-Grebe	0.035	0.0372	0.033	0.0348	0.0392	0.0346	0.1084	0.1144	0.0377	0.0772
16-Bobeat	0.0577	0.0638	0.0528	0.0557	0.0598	0.0552	0.133	0.1349	0.0739	0.1106
17-Sunnyslope	0.1551	0.1493	0.1538	0.1484	0.1585	0.135	0.2414	0.2346	0.129	0.2262
18-FondDuLac	0.1446	0.1446	0.1454	0.1505	0.1486	0.142	0.206	0.1835	0.2092	0.1974

Table 4 (concluded)

Population	Population number																	
	10	11	12	13	14	15	16	17	18									
1-Steel	0.0058	0.0043	0.0089	0.1088	<b>0.4796</b>	0.065	<b>0.2461</b>	0.1111	<b>0.5147</b>									
2-Swamp	0.0096	0.0032	-0.008	0.1059	<b>0.476</b>	0.0669	<b>0.2541</b>	0.1188	<b>0.5037</b>									
3-Wisdom	0.0128	0.0069	0.0015	0.0664	<b>0.4335</b>	0.0373	<b>0.2</b>	0.0674	<b>0.4738</b>									
4-Fishtrap	0.0097	0.0026	0.0038	0.0657	<b>0.4336</b>	0.038	<b>0.1944</b>	0.0728	<b>0.4677</b>									
5-Lamarche	0.0102	-0.0006	0.0087	0.0902	<b>0.4713</b>	0.0583	<b>0.2429</b>	0.1174	<b>0.5039</b>									
1-5-BigHole (group)	0.0129	0.0066	0.0037	0.086	<b>0.4416</b>	0.0498	<b>0.2259</b>	0.0842	<b>0.466</b>									

6-Mussigbrod	<b>0.3291</b>	0.2894	0.2896	0.4484	<b>0.7275</b>	<b>0.4499</b>	<b>0.6208</b>	0.5513	<b>0.7661</b>
7-Miner	0.0921	0.071	0.0895	<b>0.288</b>	<b>0.5945</b>	<b>0.2497</b>	<b>0.4081</b>	0.3224	<b>0.6104</b>
8-Red Rock	<b>0.1315</b>	<b>0.1299</b>	0.113	-0.0015	<b>0.3536</b>	0.0264	0.1077	0.0348	<b>0.4075</b>
9-Madison-Ennis	0.078	0.0618	0.0597	0.0956	<b>0.5021</b>	0.0868	<b>0.2964</b>	0.1448	<b>0.5521</b>
10-Ruby	-	-0.0044	0.0059	0.102	<b>0.4923</b>	0.0546	<b>0.2197</b>	0.1212	<b>0.5153</b>
11-Axolotl	0.0013 <sup>a</sup>	-	0.0037	0.0993	<b>0.4767</b>	0.06	<b>0.2319</b>	0.1242	<b>0.4953</b>
12-BFTC	0.0089	0.0107	-	0.0877	<b>0.4627</b>	0.0489	<b>0.2319</b>	0.0996	<b>0.4902</b>
13-Odell	0.0675	0.0579	0.0805	-	<b>0.3916</b>	0.0062	<b>0.1365</b>	0.0387	<b>0.4405</b>
14-Fuse	0.1578	0.1525	0.1624	0.1827	-	<b>0.4582</b>	<b>0.3486</b>	0.3814	0.0395
15-Grebe	0.0512	0.0448	0.0662	0.0162	0.1608	-	<b>0.1527</b>	0.045	<b>0.5132</b>
16-Bobcat	0.0682	0.0663	0.0783	0.045	0.191	0.037	-	0.1306	<b>0.4139</b>
17-Sunnyslope	0.1513	0.1497	0.1705	0.1234	0.2894	0.1261	0.1704	-	0.4547
18-FondDuLac	0.1624	0.156	0.166	0.1883	0.0196	0.1669	0.2022	0.3046	-

**Note:** See Table 1 for complete population names. Italics denote comparisons made among demes from within the Big Hole River (sample populations 1 through 5) that were consolidated into a single Big Hole River population ("1-5-BigHole").

Consequently, "NA" indicates estimates or tests that are not applicable because Big Hole group contains sample populations 1-

5. Bold indicates sample pairs where the allele size permutation test identified  $R_{ST} > F_{ST}$  (see text for details).

<sup>a</sup> Allele frequency tests were based on Fisher's combined probability with a modified false discovery rate (FDR) ( $n = 166$  total pairwise tests, table-wide  $\alpha = 0.05$ ) were conducted for all sample pairs. Statistical heterogeneity in allele frequencies was detected in all pairs below the diagonal except those denoted by subscript <sup>“ab”</sup>, which indicates tests where the table-wide p-values for the null hypothesis of panmixia was not rejected.

**Table 5.** Effective population sizes and genetic signatures of population bottlenecks in 14 populations of Arctic grayling (*Thymallus arcticus*).

Population	Effective population size			M-ratio bottleneck test	
	Long-term		Contemporary	$M_C$	
	$N_e$ IAM	$N_e$ SMM	$N_e$ (95% CI)	M-ratio	$\theta=1$ $\theta=10$
Native – upper Missouri River basin					
Upper Big Hole River watershed					
1-5 Big Hole River group	4045	20 411	207.6 (175.6-250.9)	0.818	0.813   0.796
6 Mussigbrod Lake	1773	4915	1496.8 (262.3-infinite)	0.747	0.802**   0.752*
7 Miner Lake	2278	7466	286.3 (142.8-4692.6)	0.787	0.801*   0.738
Red Rock lakes, Beaverhead River					
8 Red Rock Creek	2000	6000	228.2 (140.7-546.7)	0.772	0.802*   0.754
Madison River					
9 Madison River-Ennis	1423	3448	162.3 (75.5-infinite)	0.653	0.795***   0.718**
Reservoir					

Captive reserve and conservation – derived from Big Hole River							
10	Ruby River	3667	17 111	166.5 (119.4-265.6)	0.670 <sup>a</sup>	0.805***	0.754***
11	Axolotl Lake	3667	17 111	29.5 (26.3-33.3)	0.695 <sup>a</sup>	0.805***	0.760**
12	Bozeman FTC	2833	10 861	38.0 (32.8-44.5)	0.660	0.804***	0.753***
Introduced – other sources							
13	Odell Lake	2441	8400	576.9 (222.8-infinite)	0.794	0.804*	0.752
14	Fuse Lake	1500	3750	163.8 (88-694.1)	0.694	0.805**	0.752**
15	Grebe Lake	2833	10 861	-1013.7 <sup>b</sup> (1011.1-infinite)	0.753	0.803**	0.752*
16	Bobcat Lake	2000	6000	252 (114.3-infinite)	0.694	0.803***	0.752**
17	Sunnyslope Canal	851	1576	32.5 (22.9-49.3)	0.575	0.805***	0.756***
Native – Saskatchewan, Canada							
18	Fond du Lac	1583	4090	131.8 (42.8-infinite) <sup>c</sup>	0.676	0.801**	0.735**

**Notes:** Long-term effective population sizes were estimated with heterozygosity-based methods assuming either a step-wise ( $N_{eSM}$ ) or infinite allele mutation model ( $N_{eIAM}$ ), which are presumed to bound the true long-term  $N_e$ . Contemporary  $N_e$  was based on the linkage disequilibrium estimator of Waples (2006). An estimated  $M$ -ratio < the critical value ( $M_C$ ) indicates a population

bottleneck. Theta ( $\theta$ ) values of 1 and 10 correspond to pre-bottleneck effective population sizes of 500 and 5 000, respectively, assuming a microsatellite mutation rate of  $5 \times 10^{-4}$ . Symbols for statistical significance: \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , and \*\*\*  $p \leq 0.001$

<sup>a</sup> Sample populations that also exhibited genetic signatures of a population bottleneck based on heterozygosity excess included Ruby River ( $p \leq 0.001$ ) and Axolotl Lake ( $p \leq 0.01$ ).

<sup>b</sup> A negative point estimate for effective population size using program LDNe (Waples and Do 2008) means there is no evidence for any disequilibrium caused by genetic drift due to a finite number of parents, i.e.,  $N_e$  is infinite.

<sup>c</sup> Contemporary effective size estimate based on 7 loci. Three loci (Tar110, Tar114, and Tar115) were removed for analysis because of missing data.

## Figure captions

**Figure 1.** Location of Arctic grayling (*Thymallus arcticus*) sample populations. The species historical distribution in western North America (a) is shaded, and includes a disjunct set of populations that occur in the upper Missouri River basin. In the upper Missouri River (b, shaded area), grayling were native to the main stem Missouri River and most of its major tributaries (dark lines). The present range of native grayling that express a fluvial life history is restricted to the upper Big Hole River (b, dark bold line). Major dams are denoted by white bars. In the Big Hole River drainage (c), samples were collected from a number of discrete locations. In all panels, triangles indicate presumed native populations, squares denote introduced (naturalized) populations, and circles are the location of captive or introduced conservation populations derived from Arctic grayling native to the Big Hole River.

**Figure 2.** Genetic differentiation of Arctic grayling (*Thymallus arcticus*) populations from Canada, Montana, and Wyoming. Un-rooted neighbor-joining dendrogram based on Cavalli-Sforza & Edwards (1967) chord distance (CSE) calculated from allele frequencies at 10 microsatellite loci. Bootstrap probabilities, based on 1000 replicates, provide a measure of statistical confidence for each of the indicated clusters; the numbers leading to each cluster represent the percentage of times the indicated samples clustered together in the simulated, random sampling replicates. Population symbols and numbers as are in Figure 1, but with native Missouri River populations emphasized by filled triangles. Groups of populations inferred to share a common origin are indicated by hand-drawn dashed ellipses.

**Figure 3.** Mean number of alleles and mean number of private alleles per locus over 10 loci for Arctic grayling (*Thymallus arcticus*) populations in Canada, Montana, and Wyoming.

Error bars represent the standard error of the mean across loci. The location of a population on the mean number of alleles per locus axis is an indirect measure of the relative  $N_e$  among the sampled populations and its location on the mean number of private alleles per locus axis is directly related to the degree of genetic distinction that population has relative to the other sampled populations. Both measures of genetic diversity were computed according to the rarefaction method of (Kalinowski 2004) and a subsample of 36 chromosomes. See Table 1 for population numbers.

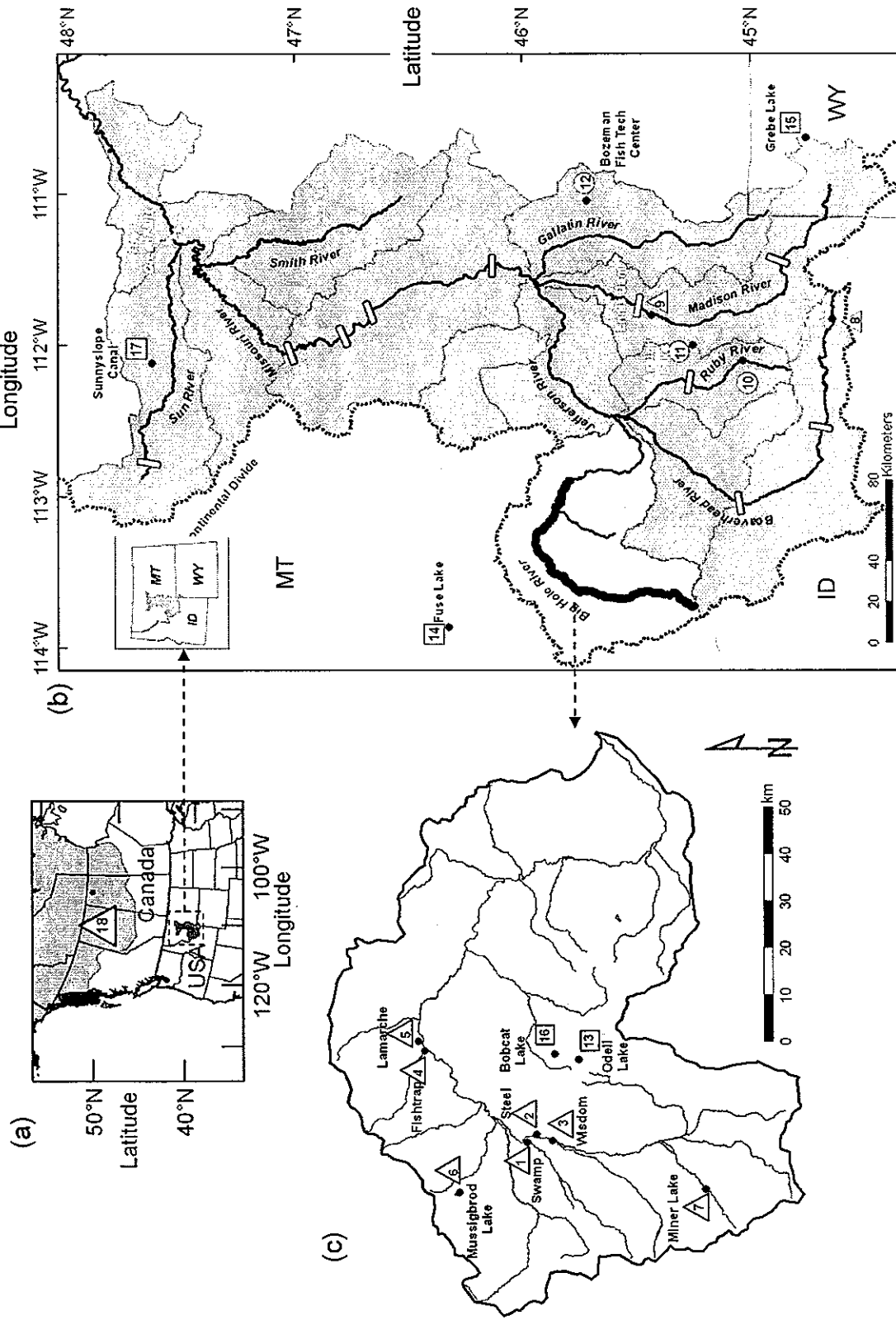


Fig 1.

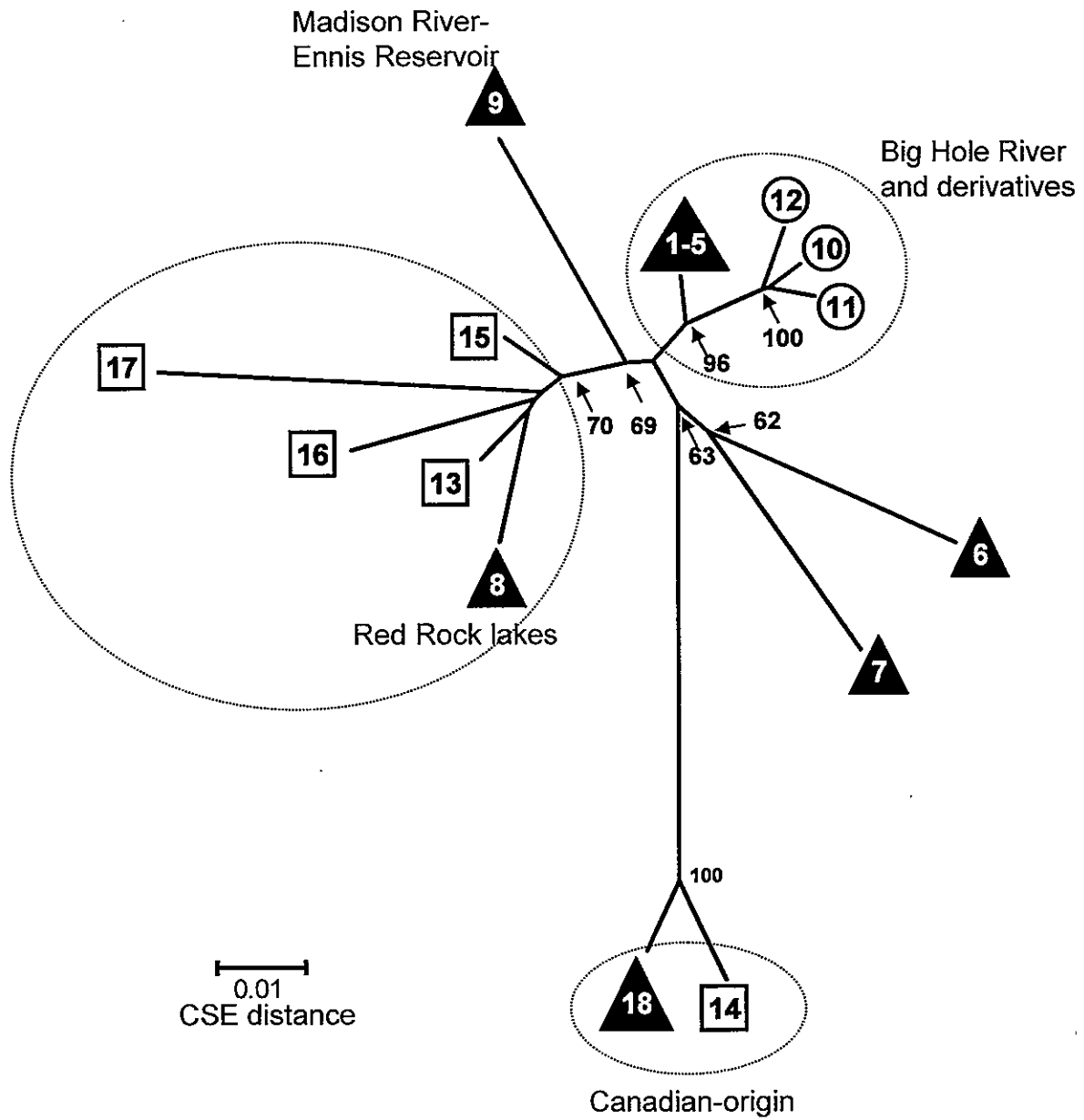


Fig 2.

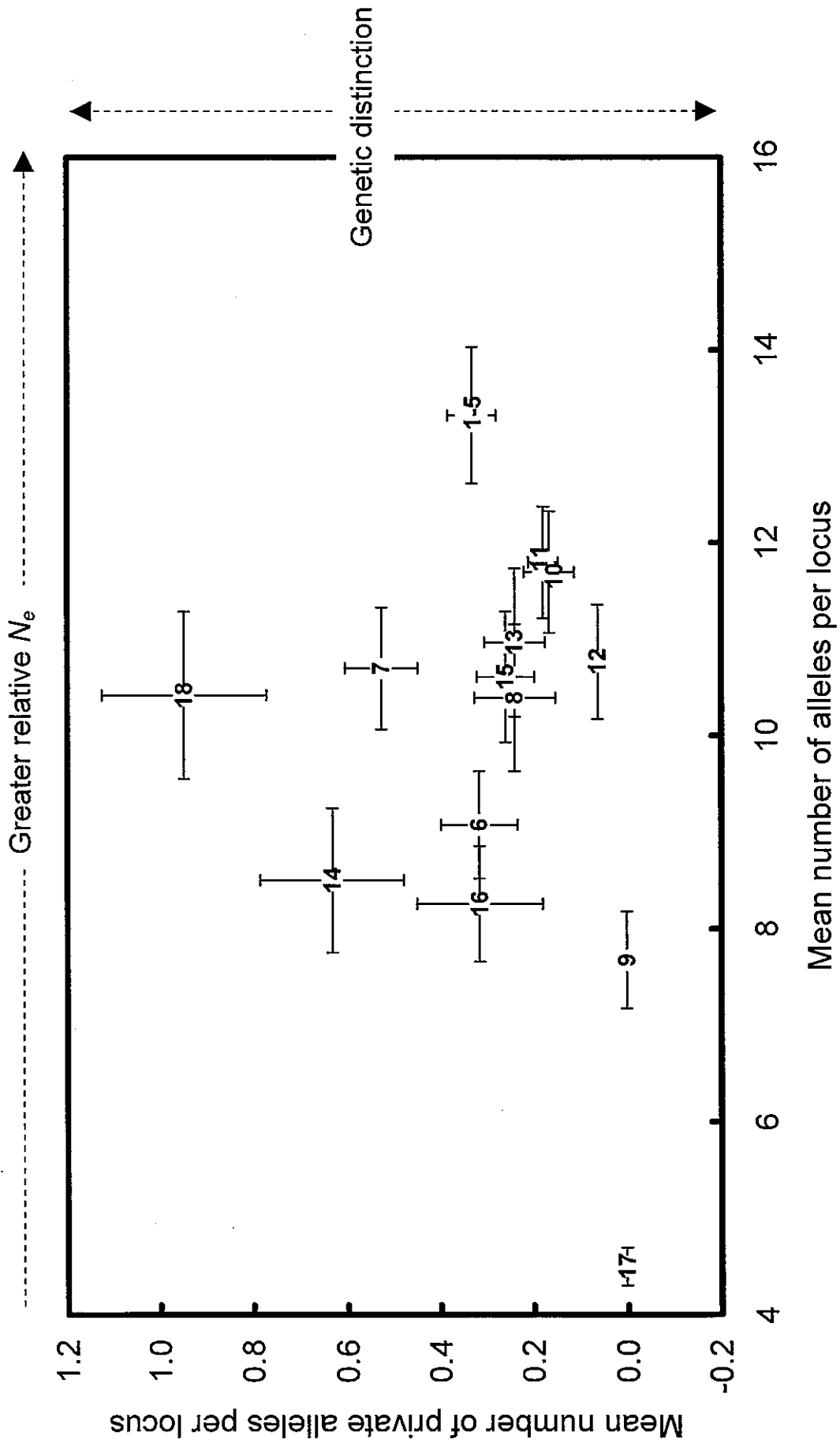


Fig. 3

