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

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Abstract: We genotyped Arctic grayling (*Thymallus arcticus*) at 10 microsatellite loci in 18 samples (n = 726) from Montana, Wyoming, and Saskatchewan to determine genetic relationships among native, captive, and naturalized populations in the upper Missouri River basin, to assess patterns in genetic diversity, and to infer recent demographic histories. Substantial genetic subdivision was observed among sample populations (global F_{ST} = 0.10). Canadian populations have been isolated from Missouri River populations long enough for mutation to cause genetic differences between regions (mean pairwise F_{ST} = 0.18, R_{ST} = 0.54). Within the Missouri River basin, most naturalized lacustrine populations traced their ancestry to Red Rock lakes. Two populations in headwater lakes within the Big Hole River watershed appear to be native. We found neither evidence for introgression of Canadian-origin grayling nor any effect of hatchery stocking in native populations. The native fluvial Big Hole River group was genetically distinct and most diverse (H_E = 0.89), whereas native Madison River and Red Rock lakes populations exhibited lower genetic diversity (H_E = 0.74 and 0.80, respectively) and evidence of recent bottlenecks. The existing Big Hole and Red Rock populations are at low abundance but do not appear to be at immediate risk of inbreeding depression (N_e = 207.7–228.2).

Résumé : Nous avons déterminé le génotype d'ombres arctiques (*Thymallus arcticus*) à 10 locus microsatellites dans 18 échantillons (n = 726) provenant du Montana, du Wyoming et de la Saskatchewan afin de déterminer les relations génétiques entre les populations indigènes, captives et naturalisées dans le bassin supérieur de la rivière Missouri, d'évaluer les patrons de diversité génétique et de déduire leur histoire démographique récente. Il existe une importante subdivision génétique des populations échantillonnées (F_{ST} global = 0,10). Les populations canadiennes ont été isolées assez longtemps des populations de la rivière Missouri pour que des mutations produisent des différences génétiques entre les régions (F_{ST} apparié moyen = 0,18, R_{ST} = 0,54). Au sein du bassin versant du Missouri, la plupart des populations lacustres naturalisées retracent leur origine aux lacs Red Rock. Deux populations dans des lacs d'amont du bassin versant de la rivière Big Hole semblent être indigènes. Il n'y a aucune indication d'introgression de la part d'ombres arctiques d'origine canadienne, ni d'effet de l'empoissonnement à partir des piscicultures sur les populations indigènes. Le groupe indigène fluvial de la Big Hole est distinct génétiquement et très diversité (H_E = 0,89), alors que les populations indigènes de la Madison et des lacs Red Rock possèdent une diversité génétique plus basse (respectivement H_E = 0,74 et 0,80) et montrent des signes de goulots d'étranglement récents. Les populations actuelles de Big Hole et de Red Rock ont une abondance faible, mais ne semblent pas courir à court terme de risque de dépression par consanguinité (N_e = 207,7 – 228,2).

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Introduction

Understanding population-level consequences of patterns in neutral genetic variation has become a preliminary step in the development of conservation programs for many commercially exploited or sensitive species. In addition to helping determine ancestral relationships and phylogeny, these patterns are commonly used to infer genetic connectivity among demes and help define populations (Waples and Gaggiotti 2006), the logical focus of conservation and management (Morris and Doak 2002). Microsatellite DNA markers can help identify cryptic population structure (e.g., So et al. 2006) and reconstruct demographic histories for rare species (e.g., Nielsen et al. 1999). Genetic-based methods can both characterize and distinguish between historical and contemporary factors that influence the demographic histories of extant populations (Pearse and Crandall 2004), for example, glacial events across tens of thousands of years (Bernatchez and Wilson 1998) versus recent habitat fragmentation, human exploitation, nonnative species invasions,

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or other impacts over tens or hundreds of years (e.g., Koskinen et al. 2002*a*; Stamford and Taylor 2005). These properties make microsatellite markers particularly amenable to understanding the conservation status of northern freshwater fishes, e.g., Arctic grayling, (*Thymallus articus*), that have been strongly influenced by both types of factors.

Arctic grayling are stenothermic cold-water, salt-water-intolerant salmonids. They are native to Arctic Ocean drainages of Alaska and northwestern Canada, as far east as Hudson's Bay, and westward across northern Eurasia to the Ural mountains (Scott and Crossman 1973; Froufe et al. 2005; Weiss et al. 2006). In North America, they are also native to northern Pacific Ocean drainages as far south as the Stikine River in British Columbia. To the south and east of the main species distribution, two disjunct groups of Arctic grayling are native to the coterminous United States: one in Michigan (extirpated in the late 1930s; Hubbs and Lagler 1949), and one in the upper Missouri River basin in Montana and Wyoming (extant). The Michigan and Missouri River populations were isolated from northern drainages by Wisconsin glaciation (Pielou 1991), but the latter apparently began to diverge genetically before that time (i.e., >70 000 years ago; Redenbach and Taylor 1999). Glacial history and genetic data suggest that the Missouri River population was founded from individuals that survived Pleistocene glacial advance in a refuge in the upper Missouri River system or southwestern Alberta, or in both places (Redenbach and Taylor 1999; Stamford and Taylor 2004).

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

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

Existing genetic data support the long-term geographic isolation of Arctic grayling from the upper Missouri River (Everett 1986; Redenbach and Taylor 1999; Stamford and Taylor 2004). These same data provide less insight into ancestral relationships among the extant native and introduced populations within the Missouri River system. In general, allozymes may exhibit less variation in recently diverged populations (Hedrick 1999), which may explain why protein electrophoresis has proven only moderately informative for distinguishing among Arctic grayling populations in Montana and Wyoming (Everett 1986; Campton 2006; R. Leary, Montana Fish, Wildlife and Parks, University of Montana, Division of Biological Sciences, Missoula, MT 59812, USA, unpublished data, 2005). The existing mtDNA data are uninformative within this region, as 24 of 25 individuals from the Big Hole River, Red Rock lakes, and Madison River populations had the same composite haplotype (Redenbach and Taylor 1999). In contrast, a genetic signature of population structure is often detected at the basin or subbasin scales for philopatric freshwater fishes such as salmonids (e.g., Costello et al. 2003; Hendry et al. 2004). Indeed, Hop and Gharrett (1989) used electrophoresis to determine the population structure of Arctic graying in the Chena River, Alaska, and studies of European grayling (*Thymallus thymallus*) have found significant population structuring within a river system (e.g., Weiss et al. 2002; Gum et al. 2003; Meldgaard et al. 2003) or even within the same lake (Koskinen et al. 2001). Fine-scale genetic analyses of North American Arctic grayling using microsatellites are few (see Stamford and Taylor 2005) but may help answer important conservation questions.

Our general objective was to use microsatellite markers to more clearly characterize ancestral relationships and genetic differentiation among native and naturalized Arctic grayling populations in the upper Missouri River. Specifically, we used these markers to infer the genetic origin of introduced populations, to determine if significant admixture between indigenous and introduced fish has resulted from past stocking, and to characterize the population structure among native populations at the basin scale (i.e., upper Missouri River). At the subbasin scale within the Big Hole River, we sought to establish whether the fluvial population functions genetically as a single, panmictic population or as a set of demes linked by occasional gene flow. For the supported native population groupings, we used genetic data to deduce their demographic histories and identify if, and when, abrupt population declines have occurred. Results are discussed rel-

ative to the genetic status and conservation of native populations with special emphasis on the Big Hole River and continuing efforts to restore fluvial populations in indigenous waters within the upper Missouri River basin.

Materials and methods

Study area and sample collection

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

Native or putative native populations and derived conservation populations

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

Waters containing populations 1–9 were extensively stocked with grayling, primarily from the Washoe Park Hatchery (Table 2). Kelly (1931) reported that the Washoe Park (Anaconda) Hatchery and a population in nearby Georgetown Lake were founded with grayling eggs from Red Rock lakes (Elk Springs Creek) and the Madison River (Meadow Creek). The Bozeman National Fish Hatchery population was founded from Red Rock lakes grayling (Kelly 1931).

The Ruby River (10), Axolotl Lake (11), and Bozeman Fish Technology Center (12) samples are derived from Big Hole River grayling taken into captivity for conservation purposes. The Ruby River has been stocked with captive-spawned juvenile graying in an attempt to re-establish a fluvial population (MFWP 2004, 2007). Axolotl Lake is a closed-basin lake that contains a brood reserve established 20 years ago (MFWP 2007). Grayling (manually) spawned from Axolotl Lake have been transplanted to the Ruby River and elsewhere in the upper Missouri River system (MFWP 2007). The Bozeman Fish Technology Center was a hatchery genetic reserve established between 1988 and 1992 (M. Toner, US Fish and Wildlife Service, Bozeman Fish Technology Center, 4050 Bridger Canyon Road, Bozeman, MT 59715, USA, personal communication).

Introduced and Canadian-origin populations

Odell (13) and Bobcat lakes (16) are small (<15 ha) headwater lakes in the Wise River system, a major tributary to the Big Hole River (Fig. 1). We could not locate any stocking records for these locations (Table 1). Grebe Lake (15) is a headwater lake (<40 ha) in the Madison River system within Yellowstone National Park. Grebe Lake is isolated by a natural geologic barrier, but grayling were introduced in 1921 and the lake became a major source of grayling eggs shipped throughout western US (Kaya 2000). Sunnyslope Canal (17) contains an introduced population derived from the stocking of its source reservoir between 1937 and 1943 (Barndt and Kaya 2000).

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

Samples from most locations consisted of fin clips collected from adults representing multiple cohorts. Native grayling were so rare in the Big Hole and Madison rivers that samples were pooled across years (Table 1) and generally included all available individuals, including young-of-the-year when present. Swamp Creek and Wisdom samples were primarily young-of-the-year from two different cohorts because adults were not present. Only young-of-the-year were present at the time of sample collection in Steel Creek, so we systematically selected a subsample of individuals for genotyping to reduce the probability of including full siblings. Scales were the DNA source for the 1996 Madison River – Ennis Reservoir samples (9) and the entire Fond du Lac sample (18).

DNA extraction and genotyping of microsatellite loci

For genomic DNA extraction, a 1 mm^2 piece of fin tissue or a single scale was placing in 200 μL of 5% chelex containing 0.2 $mg \cdot mL^{-1}$ of proteinase K; the mixture was incubated for 2 h at 56 °C, boiled for 8 min at 100 °C, and vortexed for 30 s. We used 2 μL of the supernatant in 15 μL PCR reactions to amplify 10 microsatellite loci (Table 3). Conditions for polymerase chain reaction (PCR) amplification, primer sequences, and methods for determining multilocus genotypes for all loci are reported in Diggs and Ardren (2008).

Data analyses

Within-population diversity

Average population gene diversities (H_E; Nei 1987) and allelic richness for each locus were estimated using the program HP-RARE 1.0 (Kalinowski 2005). Allelic richness was estimated using a rarefaction procedure to account for unequal sample sizes (Kalinowski 2004). The allele permutation test in FSTAT 2.9.3.2 (Goudet 2001) was used to test for differences between groups of populations in the mean values of allelic richness and H_E. Conformance of genotypic frequencies to Hardy–Weinberg expectations (HWE) was evaluated using the methods of Guo and Thompson (1992) via the program GENEPOP 4.0.7 (Raymond and Rousset 1995). Tests for genotypic disequilibrium at all pairs of loci in each sample were also calculated using GENEPOP 4.0.7, with 10 000 dememorizations, 10 000 batches, and 10 000 iterations per batch. Statistical significance levels used to detect deviations from HWE or genotypic equilibrium ratios were adjusted for the number of simultaneous tests by the sequential Bonferroni correction (Rice 1989).

Sample collections containing close relatives have the po-

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[a]	45.66682	–113.46601	1826	27	2006
2	Swamp Creek[a]	45.64978	–113.44271	1823	38	2005, 2006
3	Big Hole River near Wisdom, Montana[a]	45.61755	–113.45758	1844	22	2006
4	Fishtrap Creek[a]	45.8697	–113.22847	1779	28	2005, 2006
5	Lamarche Creek[a]	45.8834	–113.20211	1780	29	2005, 2006
Upper Big Hole River watershed						
6	Mussigbrod Lake[b]	45.79078	–113.61149	1979	48	2006
7	Miner Lake[b]	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

[a]Putative demes for fluvial Arctic grayling within the Big Hole River and connected tributaries.
[b]Putative remnant populations presently isolated by habitat degradation and water development.

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

Population structure

We used contingency tests of allele frequency homogeneity to test the null hypothesis of panmixia between any pair of sample collections. Contingency tests were conducted using the methods of Raymond and Rousset (1995) as implemented in GENEPOP 4.0.7. For each locus, we estimated the exact probability that the observed allele frequencies were drawn from the same population using the Markov chain Monte Carlo (MCMC) methods to provide an unbiased estimate of the exact probability for each randomization test. We ran 100 batches of 5000 replicates each, with 10 000 dememorisation steps. For each comparison, we used the Fisher's combined probability test (χ^2_F) as a composite test over all loci and the false discovery rate (FDR) method of Narum (2006) to set a experiment-wise α at the 0.05 level that accounts for multiple tests.

The level of genetic variation among populations was estimated by F_{ST} using the θ statistic of Weir and Cockerham (1984). Pairwise F_{ST} values were estimated in GENEPOP 4.0.7. Estimates of F_{ST} over all sample locations and associated 95% confidence intervals, generated by bootstrap sampling over loci, were calculated in FSTAT 2.9.3.2 (Goudet 2001). Testing for different ancestral histories between populations was accomplished by comparing global variance in allelic identity (i.e., F_{ST}) and allelic size (i.e., R_{ST}) measures of genetic difference. Under the null hypothesis ($R_{ST} = F_{ST}$), differentiation is caused mainly by drift, whereas under the alternative hypothesis ($R_{ST} > F_{ST}$), stepwise mutations have contributed to differentiation (Hardy et al. 2003). SPAGEDI

Fig. 1. Location of Arctic grayling (*Thymallus arcticus*) sample populations. (*a*) The species historical distribution in western North America is shaded and includes a disjunct set of populations that occur in the upper Missouri River basin. (*b*) In the upper Missouri River, grayling were native to the main stem Missouri River (shaded area) 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 (dark bold line). Major dams are denoted by white bars. (*c*) In the Big Hole River drainage, 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.

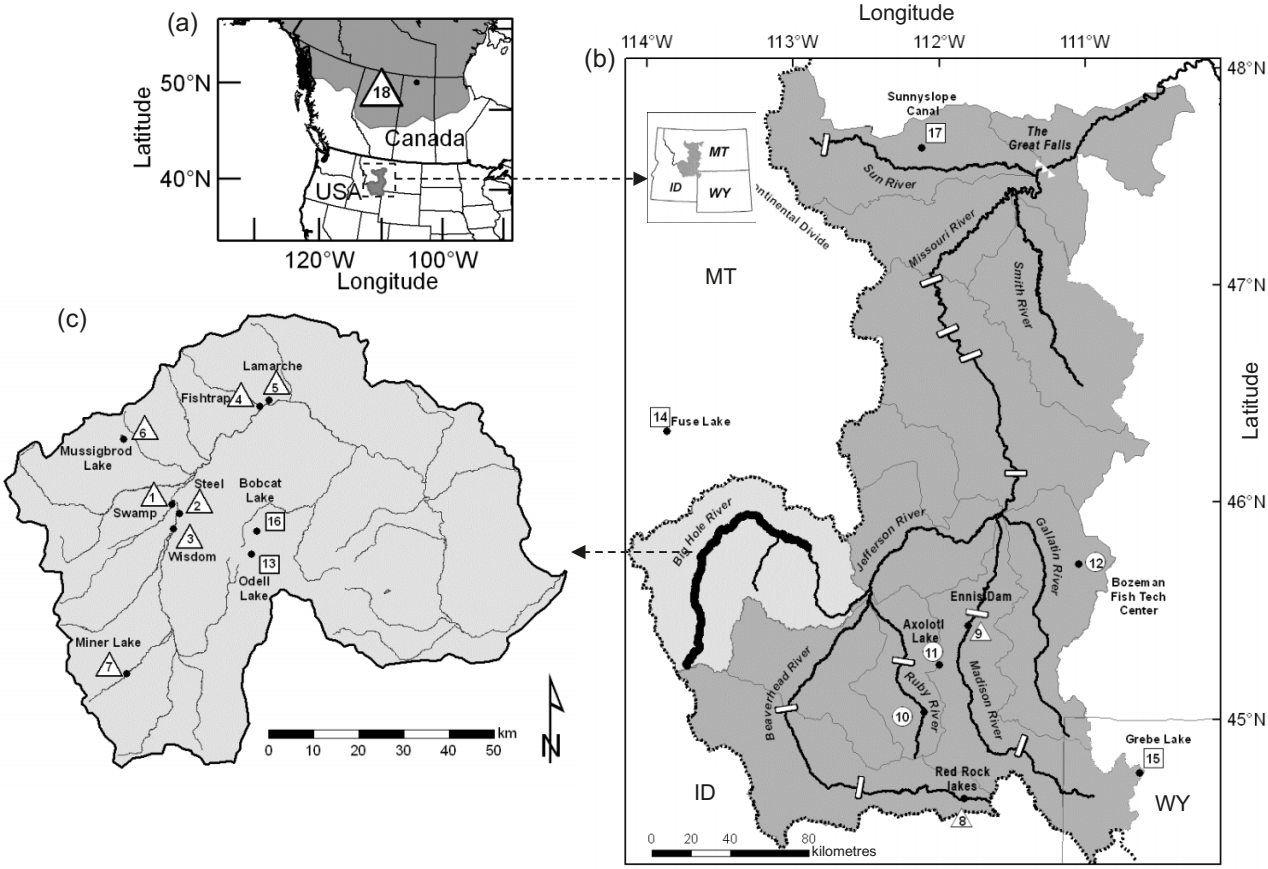


Table 2. Documented stocking of hatchery-cultured Arctic grayling (*Thymallus arcticus*) into waters containing indigenous or presumed indigenous populations.

No.	Population	Years stocked	Total number stocked	Hatchery source
1–5	Big Hole River group	1937–1957	14 732 193[a]	Washoe Park Hatchery (MFWP 2005*b*)[b]
6	Mussigbrod Lake	1934–1955	5 951 847	Washoe Park Hatchery (MFWP 2005*b*)[b]
7	Miner Lake	1933–1952	3 220 776	Washoe Park Hatchery (MFWP 2005*b*)[b]
8	Red Rock	1899–1938	Unknown	Bozeman National Fish Hatchery (Randall 1978)
		1946–1962	103 704	Ennis National Fish Hatchery (Randall 1978; MFWP 2005*b*)
9	Madison River – Ennis Reservoir	1928–1961	2 690 725[c]	Washoe Park during 1928–1934, Ennis National Fish Hatchery during 1946–1962 (MFWP 2005*b*)

[a]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.

[b]The Washoe Park Hatchery is operated by the state of Montana and was historically referred to as the "Anaconda Hatchery".

[c]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 2005*b*).

v1.2 (Hardy and Vekemans 2002) was used to calculate R_{ST} and to test for significant differences between F_{ST} and R_{ST} using the allele size permutation test of Hardy et al. (2003) (α = 0.05 with FDR adjustment). We used standard error estimates from SPAGEDI v1.2 to calculate 95% confidence intervals for the average pairwise R_{ST} estimates over all populations. To test for geographic structure and consistency with a stepping-stone model of gene flow among native pop-

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

No.	Sample population	N	N_A	N_A^{36}	N_P	H_O	H_E	HWE	LD	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[a]	144	19.3	13.3[b]	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 multilocus genotypes (after Belkhir et al. 2002), where values range from 0 for unrelated individuals to 0.5 for full siblings.

[a]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).

[b]Table value for allelic richness estimated using HP-RARE 1.0. Allelic richness estimated using FSTAT 2.9.3.2 was slightly lower (N_A^{36} = 13.24; see Results, Within population diversity).

ulations with presumed historical connectivity, we examined the correlation between the natural log of pairwise fluvial distances (km) and genetic differentiation using Mantel tests implemented in GENEPOP 3.7 (Rousset 1997).

Genetic relationships among populations were inferred by generating an unrooted neighbor-joining (NJ) tree based on Cavalli-Sforza and Edwards' (1967) chord distance. Confidence in the observed topology of the NJ–population tree was assessed using the bootstrap procedure in PHYLIP v3.6 (Felsenstein 1992) based on 1000 resamples across loci. The consensus NJ–population tree was generated in PHYLIP v3.6.

Effective population size

We estimated the long-term effective population size (N_e) using the heterozygosity-based methods of Ohta and Kimura (1973) (eq. 1) and Hartl and Clark (1989) (eq. 2). Both methods assume selective neutrality and predict that at mutation-drift equilibrium, N_e is a function of H_E. Ohta and Kimura (1973) assume a stepwise mutation model (SMM), whereas Hartl and Clark (1989) assume an infinite allele mutation model (IAM). We used the most commonly applied microsatellite mutation rate for fishes, $\mu = 5 \times 10^{-4}$ (Estoup and Angers 1998).

$$(1) \qquad N_{\mathrm{eSMM}} = \frac{\left[\left(\frac{1}{1-H_E}\right)^2 - 1\right]}{8\mu}$$

$$(2) \qquad N_{\mathrm{eIAM}} = \frac{H_E}{4\mu(1-H_E)}$$

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

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

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

		Population number								
No.	Population	1	2	3	4	5	1–5	6	7	8
1	Steel	—	*–0.0096*	*–0.0083*	*–0.0047*	*–0.0063*	NA	**0.3536**	0.0859	**0.1364**
2	Swamp	*0.0036* a	—	*0.0004*	*–0.0031*	*–0.0106*	NA	0.2905	0.0694	**0.1326**
3	Wisdom	*–0.0027* a	*0.0019* a	—	*–0.0125*	*–0.0054*	NA	**0.381**	0.1167	0.0897
4	Fishtrap	*0.0052*	*0.0019* a	*–0.0052* a	—	*–0.0118*	NA	0.3488	0.0995	0.0861
5	Lamarche	*0.0053*	*0.0055* a	*–0.0019* a	*–0.003* a	—	NA	**0.291**	0.0755	0.116
1–5	Big Hole group	NA	NA	NA	NA	NA	—	**0.2756**	0.086	0.1112
6	Mussigbrod	0.0789	0.0805	0.0772	0.0743	0.0719	0.0719	—	**0.3075**	**0.4784**
7	Miner	0.0789	0.072	0.072	0.0728	0.0777	0.072	0.1199	—	**0.3172**
8	Red Rock	0.0808	0.0747	0.0699	0.0694	0.0782	0.0708	0.1604	0.1501	—
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
11	Axolotl	0.0232	0.0155	0.0134	0.0138	0.0182	0.0162	0.0981	0.0758	0.0734
12	BFTC	0.0318	0.0233	0.024	0.0237	0.0293	0.025	0.1044	0.086	0.0929
13	Odell	0.0537	0.0579	0.0492	0.0464	0.0568	0.0508	0.1389	0.1329	0.0222
14	Fuse	0.1375	0.1362	0.1399	0.1404	0.1414	0.1301	0.1962	0.1862	0.2021
15	Grebe	0.035	0.0372	0.033	0.0348	0.0392	0.0346	0.1084	0.1144	0.0377
16	Bobcat	0.0577	0.0638	0.0528	0.0557	0.0598	0.0552	0.133	0.1349	0.0739
17	Sunnyslope	0.1551	0.1493	0.1538	0.1484	0.1585	0.135	0.2414	0.2346	0.129
18	Fond du Lac	0.1446	0.1446	0.1454	0.1505	0.1486	0.142	0.206	0.1835	0.2092

Note: See Table 1 for complete population names. Allele frequency tests, based on Fisher's combined probability with a modified false discovery rate detected in all pairs below the diagonal, except those followed by the letter "a", which indicates tests in which the tablewide p values for the null hypothesis that were consolidated into a single Big Hole River population (1–5, Big Hole group). Consequently, NA indicates estimates or tests that are not applicable tified $R_{ST} > F_{ST}$.

Tests for recent population declines

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

Tests for heterozygosity excess were done with the program BOTTLENECK v1.2.02 (Piry et al. 1999) using a two-phase model (TPM) of microsatellite mutation (Di Rienzo et al. 1994) with parameter settings of 95% SMM, 5% IAM, and 12% variance in multistep mutations (i.e., presumed model for microsatellites; Piry et al. 1999; Lippé et al. 2006). Significance of heterozygosity excess observed in a population was evaluated by a one-sided Wilcoxon's signed-rank test ($\alpha = 0.05$) testing the null hypothesis of no significant heterozygosity excess based on 5000 simulation iterations. M ratios were calculated in M_P_VAL (Garza and Williamson 2001). The M ratio is a measure of the number of alleles (k) to the allele size range (r). The M ratio (k/r) is expected to be small in a recently bottlenecked population because k is reduced faster than r. The program M_P_VAL implemented a model of microsatellite evolution assuming 88% one-step mutations (p_g) and 2.8 average-sized non-one-step mutations (Δ_g) (Garza and Williamson 2001). We explored a range of sizes for the prebottlenecked population (N_e = 500 and 5000) and a microsatellite mutation rate of $\mu = 5 \times 10^{-4}$ to estimate θ ($= 4 \times N_e \times \mu$). Statistical significance of the observed M ratio was calculated using the program Critical_M (Garza and Williamson 2001), with the critical value (M_c) for each sample such that 95% of 10 000 simulations of an equilibrium population had M ratio > M_c.

Results

Within-population diversity

We observed moderate to high levels of genetic diversity in Arctic grayling across all 10 microsatellite loci. Expected heterozygosites (H_E) over all loci ranged from 0.63 in Sunnyslope Canal to 0.89 in four Big Hole River samples (Table 3; see also Supplemental Table S1[3]) and averaged 0.80 across all samples The number of alleles per locus (N_A) ranged from 5.0 in Sunnyslope Canal to 16.7 in

[3] Supplementary data for this article are available on the journal Web site (http://cjfas.nrc.ca) or may be purchased from the Depository of Unpublished Data, Document Delivery, CISTI, National Research Council Canada, Building M-55, 1200 Montreal Road, Ottawa, ON K1A 0R6, Canada. DUD 5273. For more information on obtaining material, refer to http://cisti-icist.nrc-cnrc.gc.ca/eng/ibp/cisti/collection/unpublished-data.html.

loci.

9	10	11	12	13	14	15	16	17	18
0.0628	0.0058	0.0043	0.0089	0.1088	**0.4796**	0.065	**0.2461**	0.1111	**0.5147**
0.0638	0.0096	0.0032	–0.008	0.1059	**0.476**	0.0669	**0.2541**	0.1188	**0.5037**
0.0547	0.0128	0.0069	0.0015	0.0664	**0.4335**	0.0373	**0.2**	0.0674	**0.4738**
0.0509	0.0097	0.0026	0.0038	0.0657	**0.4336**	0.038	**0.1944**	0.0728	**0.4677**
0.0586	0.0102	–0.0006	0.0087	0.0902	**0.4713**	0.0583	**0.2429**	0.1174	**0.5039**
0.0506	0.0129	0.0066	0.0037	0.086	**0.4416**	0.0498	**0.2259**	0.0842	**0.466**
0.4246	**0.3291**	0.2894	0.2896	0.4484	**0.7275**	**0.4499**	**0.6208**	0.5513	**0.7661**
0.1975	0.0921	0.071	0.0895	**0.288**	**0.5945**	**0.2497**	**0.4081**	0.3224	**0.6104**
0.1442	**0.1315**	**0.1299**	0.113	–0.0015	**0.3536**	0.0264	0.1077	0.0348	**0.4075**
—	0.078	0.0618	0.0597	0.0956	**0.5021**	0.0868	**0.2964**	0.1448	**0.5521**
0.0987	—	–0.0044	0.0059	0.102	**0.4923**	0.0546	**0.2197**	0.1212	**0.5153**
0.0938	0.0013 a	—	0.0037	0.0993	**0.4767**	0.06	**0.2319**	0.1242	**0.4953**
0.1023	0.0089	0.0107	—	0.0877	**0.4627**	0.0489	**0.2319**	0.0996	**0.4902**
0.1246	0.0675	0.0579	0.0805	—	**0.3916**	0.0062	**0.1365**	0.0387	**0.4405**
0.1876	0.1578	0.1525	0.1624	0.1827	—	**0.4582**	**0.3486**	0.3814	0.0395
0.0772	0.0512	0.0448	0.0662	0.0162	0.1608	—	**0.1527**	0.045	**0.5132**
0.1106	0.0682	0.0663	0.0783	0.045	0.191	0.037	—	0.1306	**0.4139**
0.2262	0.1513	0.1497	0.1705	0.1234	0.2894	0.1261	0.1704	—	0.4547
0.1974	0.1624	0.156	0.166	0.1883	0.0196	0.1669	0.2022	0.3046	—

(FDR) (n = 166 total pairwise tests, tablewide α = 0.05), were conducted for all sample pairs. Statistical heterogeneity in allele frequencies was of panmixia was not rejected. Italics denote comparisons made among demes from within the Big Hole River (sample populations 1 through 5) because the Big Hole group contains sample populations 1–5. Bold type indicates sample pairs in which the allele size permutation test iden-

Swamp Creek (a tributary to the Big Hole River) and averaged 12.7 across all samples. The Big Hole River group exhibited higher allelic richness, using a sample of 36 genes (N_A^{36} = 13.24) and H_E (0.89), than the other native populations combined (i.e., populations 6–9; N_A^{36} = 9.46 and H_E = 0.79, $p < 0.05$ for both comparisons).

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

Individuals within sample populations did not appear to be highly related to one another, with a few exceptions. None of the populations violated the test assumption of being more related than expected based on a random draw of individuals from a panmictic population (lowest one-sided p value = 0.053, average p value = 0.56, SD = 0.32). Most populations had relatedness coefficients (I) less than 0.25 (i.e., analogous to half siblings; Table 3). Fish sampled in the Big Hole River were less related on average (mean I = 0.14) than fish sampled from the other native Missouri River populations (mean I = 0.25). The Sunnyslope Canal was nearly consistent with a population of full siblings (I = 0.49).

Population structure

We observed substantial genetic subdivision among grayling populations outside of the connected Big Hole River (Table 4). Pairwise F_{ST} estimates generally mirrored the differences in allele frequencies: genetic differentiation among demes within the connected Big Hole River was minimal ($F_{ST} \leq 0.0055$, homogeneity in allele frequencies in 8 of 10 comparisons), whereas differentiation between the Big Hole River group (i.e., populations 1–5) and all other wild native and naturalized populations was greater ($F_{ST} \geq 0.035$, heterogeneity in allele frequencies in 10 of 10 cases). The greatest differentiation was generally observed between Canadian-origin populations (Fuse Lake, Fond du Lac) and those from the Missouri River system (range F_{ST} = 0.13–0.31). Canadian-origin populations contained many alleles not found in any of the Montana populations, a high frequency of private alleles at six loci, and a very different distribution of allele size ranges (see Supplemental Table S2[3]). Pairwise F_{ST} values between the five native grayling populations in Montana (populations 1–5, 6, 7, 8, and 9) ranged from at least 0.071 between the combined Big Hole River group (1–5) and all other populations, to 0.174 between the Red Rock lakes (8) and the Madison River – Ennis Reservoir (9).

The global R_{ST} of 0.25 (95% CI 0.17–0.33; n = 14 populations (group 1–5 and populations 6–18)) was much larger than the corresponding global F_{ST} of 0.10 (95% CI 0.07–0.15), indicating that F_{ST} underestimates the actual levels of genetic structure in some comparisons. The allele size permutation test identified 41 of 91 pairwise comparisons of populations in which $R_{ST} > F_{ST}$ (Table 4; the 91 comparisons exclude individual estimates for sample sites within the connected Big Hole River group (populations 1, 2, 3, 4, and 5). Pairwise R_{ST} values were largest between Canadian-origin populations (14, 18) and the upper Missouri River populations (1–9), with the largest value (R_{ST} = 0.76) observed between Mussingbrod Lake (6) and Fond du Lac (18). Mussingbrod Lake (6) was the most genetically distinct

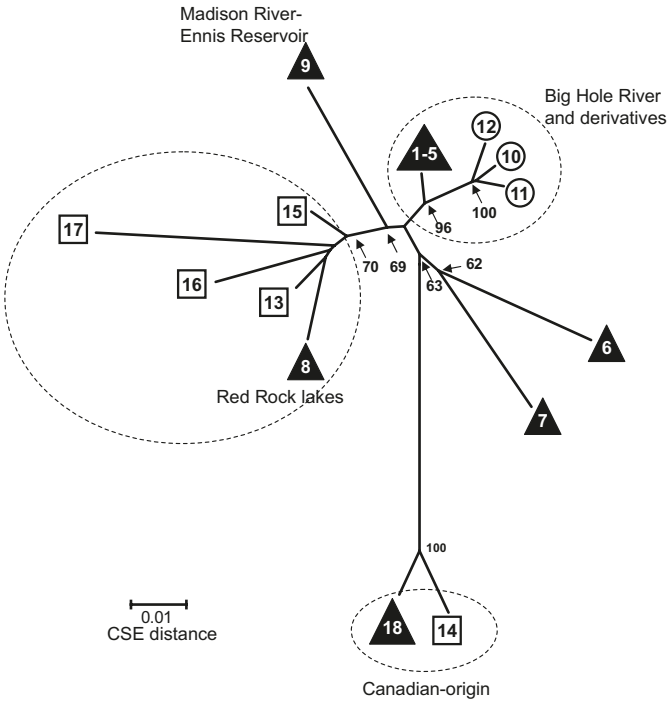
among the native Missouri River populations (1–9) based on pairwise R_{ST} estimates. The introduced population in Bobcat Lake (16) had $R_{ST} > F_{ST}$ for all comparisons except when compared with Red Rock lakes (8).

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

Clusters of individuals from native grayling locations in the Missouri River (populations 1–9) identified using the Bayesian method of STRUCTURE (Pritchard et al. 2000) were consistent with patterns of population structure observed with genic tests, F_{ST}, R_{ST}, and the phylogenetic analysis (see Supplemental Table S3[3]). Evaluation of the assignment of individuals to six clusters had a clear biological interpretation based on geography and physical isolation. There was little support for population structure within the connected Big Hole River (populations 1–5); however, fish from this system did form a cluster that was distinct from the other locations sampled. Fish collected from Mussingbrod Lake (6), Miner Lake, Red Rock lakes (7), Madison River (8), and Fuse Lake (14) all formed distinct genetic clusters.

We did not detect a statistically significant isolation-by-distance effect based on based on genetic differentiation that considered mutation (R_{ST}, n = 10, Spearman's r_S = 0.164, Mantel p value = 0.35) among the five presumed native populations in the upper Missouri River system (see Supplemental Table S4[3]). Differentiation based on genetic drift (F_{ST}) did indicate isolation-by-distance (Supplemental Table S4[3]); however, F_{ST} underestimates differentiation among sample pairs that include Miner and Mussigbrod lakes, where differences are due, in part, to mutation (Table 4).

Fig. 2. Genetic differentiation of Arctic grayling (*Thymallus arcticus*) populations from Canada, Montana, and Wyoming. Unrooted neighbor-joining dendrogram based on Cavalli-Sforza and Edwards (1967) chord distance (CSE) are 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 that the indicated samples clustered together in the simulated, random sampling replicates. Population symbols and numbers as are in Fig. 1, but with native Missouri River populations emphasized by solid triangles. Groups of populations inferred to share a common origin are enclosed by ellipses.



Effective population size

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

Six populations stood out in a bivariate plot of the mean number of alleles per locus versus the mean number of private alleles per locus (Fig. 3). Both Sunnyslope Canal and Madison River had low number of alleles and low level of private alleles, suggesting that these populations have small N_e relative to the rest of the populations and that they are genetically similar to the other Montana populations. The Canadian-origin populations had the highest levels of private alleles and an intermediate level of mean number of alleles, indicating long-term genetic isolation relative to Montana populations. Among Montana populations, Miner Lake had the most private alleles per locus, suggesting that it has had a relatively low level of gene flow with the other native Montana populations. The highest number of alleles was observed in the Big Hole River group, which is consistent with this population having a comparatively large N_e.

Population bottlenecks

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

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

		Effective population size			*M*-ratio bottleneck test		
		Long-term				M_C	
No.	Population	N_{eIAM}	N_{eSMM}	Contemporary 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 Reservoir	1423	3448	162.3 (75.5–infinite)	0.653	0.795***	0.718**
Captive reserve and conservation — derived from Big Hole River							
10	Ruby River	3667	17 111	166.5 (119.4–265.6)	0.670[a]	0.805***	0.754***
11	Axolotl Lake	3667	17 111	29.5 (26.3–33.3)	0.695[a]	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[b] (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)[c]	0.676	0.801**	0.735**

Note: Long-term effective population sizes were estimated with heterozygosity-based methods assuming either a stepwise (N_{eSMM}) 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 (θ) values of 1 and 10 correspond to prebottleneck effective population sizes of 500 and 5000, respectively, assuming a microsatellite mutation rate of 5×10^{-4}. Statistical significance: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

[a]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 ($p \leq 0.01$).

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

[c]Contemporary effective size estimate based on seven loci. Three loci (Tar110, Tar114, and Tar115) were removed for analysis because of missing data.

Discussion

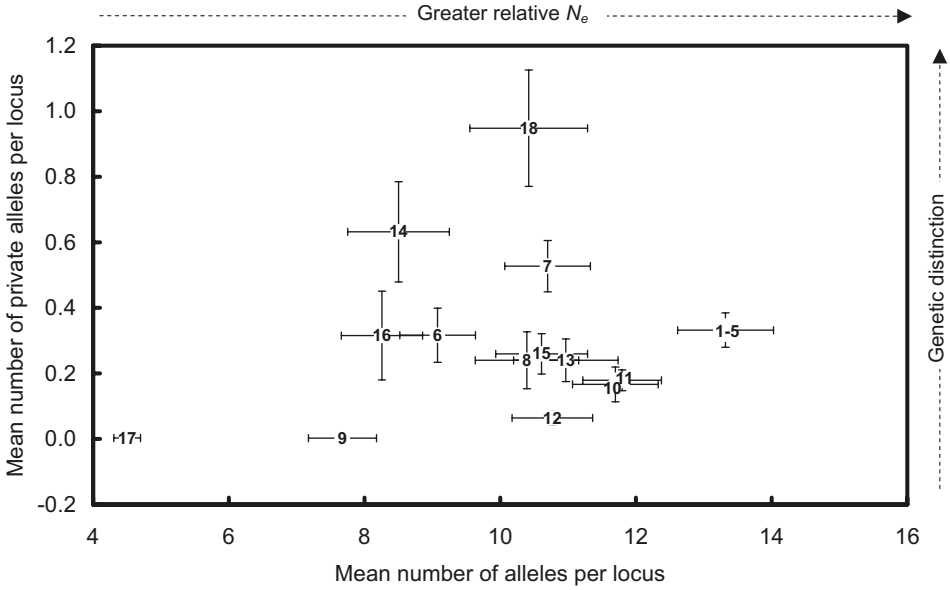
Ancestry and population structure in Missouri River Arctic grayling

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

Arctic grayling from the connected Big Hole River form a genetic group distinct from other native populations in the Madison River – Ennis Reservoir and Red Rock lakes. In turn, there is also moderate support that these latter two populations each form discrete genetic groups. Population groupings based on protein electrophoresis previously found no genetic distinction between Big Hole River and Madison River grayling (Everett 1986; R. Leary, Montana Fish, Wildlife and Parks, University of Montana, Division of Biological Sciences, Missoula, MT 59812, USA, unpublished data), perhaps because only 2 of 39 allozyme loci were polymorphic.

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

Fig. 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.



Genetic and biogeographic data suggest that Arctic grayling in Miner and Mussigbrod lakes may be remnant native populations. The level of genetic differentiation in these populations also indicates that they have been demographically independent for some time. Mussingbrod Lake had $R_{ST} > F_{ST}$ relative to the connected Big Hole River and Miner Lake, indicating that it has been isolated long enough for mutation to have contributed to its genetic distinction. In contrast, Miner Lake had $R_{ST} = F_{ST}$ relative to the connected Big Hole River, indicating that genetic drift has caused the observed differentiation and that the population has been isolated for less time than the Mussingbrod Lake population. Stocking of hatchery fish could also account for large R_{ST} values in these lakes; however, both Miner and Mussigbrod lakes were strongly differentiated from the Red Rock lakes population, with $R_{ST} > F_{ST}$ for both comparisons (F_{ST} = 0.15–0.16; R_{ST} = 0.32–0.48). In addition, Miner and Mussigbrod lakes did not cluster with other naturalized populations apparently derived from Red Rock lakes. Such differentiation would be unlikely if Red Rocks grayling were stocked in those waters and made a significant genetic contribution to the extant population. Presence of other native fishes in high-elevation lakes in the upper Big Hole River watershed (e.g., Vincent 1963) demonstrates historical connectivity with the mainstem river. Indigenous burbot (*Lota lota*) and longnose sucker (*Catostomus catostomus*) populations in Miner and Mussigbrod lakes (B. Snyder, Montana Fish, Wildlife and Parks, 1420 East Sixth Avenue, Helena, MT 59620–0701, USA, personal communication, 2006) implies that natural colonization by grayling was also possible. Consequently, we conclude that grayling populations in Miner and Mussigbrod lakes are not solely derived from stocking. The timing of the initial colonization of these lakes by grayling and other native fishes is unknown and warrants further investigation.

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

Thymallus populations influenced by intraspecific stocking sometimes exhibit greater genetic diversity than unaltered native populations because of the introduction of nonnative genes and Wahlund effects (e.g., Gum et al. 2003). The Big Hole group was the most diverse sample population, but to attribute this to admixture from stocking, one would have to discount landscape genetic arguments that greater diversity in the Big Hole population (relative to other native populations) could be a function of multiple spawning locations and greater habitat extent (e.g., Neville et al. 2006) or habitat connectivity (e.g., Costello et al. 2003; Taylor et al. 2003; Wofford et al. 2005). A direct test of introgression (e.g., Koskinen et al. 2002*b*; Gum et al. 2006) was not possible for indigenous Missouri River grayling because we did not have any samples collected before

stocking. Theoretically, stocking from different sources, or stocking from a single source following by genetic drift, could have affected the genetic characteristics of Big Hole River grayling. This appears unlikely given our data and considering that most attempts to introduce Arctic grayling into riverine habitats generally have not been successful (Northcote 1995; Kaya 2000; MFWP 2004).

Little or no recent gene flow has occurred among grayling populations in the Big Hole, Madison, and Beaverhead river subbasins or between Mussigbrod and Miner lakes in the Big Hole River watershed. In contrast, the data suggest recent genetic exchange among spawning sites within the connected Big Hole River, as the demes sampled were largely panmictic. The spatial scale of population structuring for Missouri River grayling (over hundreds of kilometres) is similar to observation of Arctic grayling from rivers in Alaska (Hop and Gharrett 1989) and British Columbia (Stamford and Taylor 2005). Landscape genetic variation in stream salmonids is driven by historical and contemporary factors such as glaciation and habitat modification (e.g., Costello et al. 2003; Taylor et al. 2003; Stamford and Taylor 2005); species-specific differences in behavior and life history (e.g., Wenburg et al. 1998; Heggenes et al. 2006; Neville et al. 2006), and ecological context (e.g., Haugen and Vøllestad 2000; Koskinen et al. 2001). Population genetic structure in *Thymallus* has been more frequently detected among subbasins (Koskinen et al. 2002*a*; Gum et al. 2003; but see Koskinen et al. 2001) relative to other salmonids that show differentiation at finer scales, such as among or within tributary streams (e.g., Carlsson et al. 1999; Taylor et al. 2003; Young et al. 2004).

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

Demographic status and population declines inferred from genetic data

Arctic grayling in the Missouri River system were historically very abundant, with long-term N_e ranging from a few thousand to tens of thousands. There is some uncertainty as to the time frame and demographic scale associated with long-term estimates of effective population size (Waples 1991), but most authors agree that these estimates can be interpreted as the harmonic mean of the N_e in each generation among populations within a geographic region (Kalinowski and Waples 2002). In this case, the long-term estimates are best interpreted as N_e for Arctic grayling populations in the Missouri basin since the Wisconsin glacier receded approximately 10 000 years ago. Contemporary N_e for individual populations was significantly less than the long-term N_e. Most populations did not exhibit inbreeding (i.e., contemporary $N_e > 50$), but N_e were low enough to raise concern about maintaining adaptive potential.

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

We expected to find a bottleneck in the Big Hole group given that the population is at low abundance. Perhaps the genetic signatures of a bottleneck are not apparent, despite a demographic decline (e.g., see Busch et al. 2007), or the decline has been more gradual or smaller compared with other native populations. Arctic grayling still spawn in multiple locations in the Big Hole River and the mainstem migratory corridor is largely intact. In contrast, fragmentation of mainstem habitats likely contributed to bottlenecks observed in Red Rock lakes and the Madison River. In the Red Rock River system, grayling were extirpated from numerous tributary streams during the last 100 years, and the remaining adfluvial population in the headwater lakes was isolated by a barrier installed in 1957 (Nelson 1954; Vincent 1962; Mogen 1996). In the Madison River, the construction of impassible Ennis Dam >100 years ago constrained, if not eliminated, any expression of the fluvial life history for grayling (Vincent 1962; Kaya 1992). The residual (adfluvial) Madison River population may have been further affected by drawdowns of Ennis Reservoir in the 1960s–1980s, which reduced surface area by up to 15%–50% (MFWP 1990).

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

Conservation implications

Arctic grayling within the connected Big Hole River should tentatively be considered a single population because we detected no, to very low levels of, molecular divergence between putative demes within that system. This apparent panmixia could be a very recent development or a consistent pattern over the past few decades (W.R. Ardren and D.P. Peterson, unpublished data). The native Big Hole, Madison, Red Rock, Miner, and Mussigbrod populations in the Missouri River basin are demographically independent from one another. Genetic approaches to defining conservation

and management units have been proposed (e.g., Crandall et al. 2000; Palsbøll et al. 2007), but anthropogenic isolation (especially in Big Hole, Madison, and Red Rock), and long-term reproductive isolation (e.g., Mussigbrod and Miner) among native populations dictates that they be managed as separate units. We presume that the native Missouri River populations were historically linked by occasional gene flow and must have exhibited some metapopulation-like structure (e.g., Rieman and Dunham 2000). In the future, facilitating habitat connectivity among subbasins occupied by native populations would help reduce genetic and stochastic risks. The challenge in doing this, however, will be considerable given existing fragmentation and habitat alteration and possible threats from further nonnative trout invasions (Fausch et al. 2009).

The Big Hole River population represents an important genetic reservoir for the species within the Missouri River basin and is also the last example of the riverine, migratory (fluvial) ecotype in the coterminous US (Kaya 1992). Genetic and life history diversity should help buffer populations against environmental variation, catastrophe, and anthropogenic stressors (e.g., Rieman and McIntyre 1993; Crandall et al. 2000; Fausch et al. 2006). By these criteria, the Big Hole River should be a high conservation priority. US state and federal agencies are working with private landowners in the upper Big Hole River basin to implement a conservation agreement to augment streamflows, restore riparian habitats, remove migration barriers, and reduce entrainment in irrigation ditches (MFWP 2006).

Efforts to use captive populations to re-establish wild fluvial populations in indigenous waters will probably continue to challenge grayling restoration biologists. Drought, nonnative trout, and a lack of imprinting by stocked fish may have limited reintroduction success of fluvial Arctic grayling in Montana (MFWP 2003, 2007). A general lack of connected, mainstem river habitats accessible to grayling may further constrain expression of a migratory life history at translocation sites. The genetic data suggest that additional factors may warrant investigation. First, genetic effects of captivity and culture may be unavoidable (e.g., Frankham 2008), and a slight reduction in genetic diversity has occurred in the ancestors of the captive population derived from Big Hole River grayling. There are no data to indicate that these observations are directly related to translocation success, but an examination of behavioral and physiological differences between wild and captive grayling (e.g., in a common-garden setting) or measurable changes in fitness (e.g., in captive or hatchery fish released into their indigenous habitats) would provide a more rigorous evaluation. Second, divergence among populations measured by molecular markers can potentially indicate differences in behavior, ecology, life history, and physiology (Stamford and Taylor 2005) that influence survival in specific habitats (Merilä and Crnokrak 2001; e.g., in *Thymallus*, see Koskinen et al. 2001; Salonen 2005). If genetic differentiation in the Big Hole River group is concordant with local adaptation (Taylor 1991), then a lack of ecological exchangeability (sensu Crandall et al. 2000) between subbasins may, in part, explain why translocations using Big Hole River grayling have yet to establish a self-sustaining population in another river. Common-garden experiments that test for unique or population-specific traits would provide insight into local adaptation. A systematic evaluation of factors influencing translocation success that also considers downscaled climate projections could generate spatially explicit decision support models to help guide future translocation efforts.

Although introductions using Big Hole River stock have not yet produced a self-sustaining fluvial population, few alternatives exist for ongoing translocations. Adfluvial Arctic grayling have not been successfully transplanted into rivers in Montana (Kaya 1992), and heritable behavioral differences exist between adfluvial and fluvial stocks (e.g., Kaya 1991; Kaya and Jeanes 1995). Madison River grayling are extremely rare (MFWP 2007), no brood reserve exists for the population, and the contemporary N_e (162.3) is based mostly on fish captured one or two generations ago. Moreover, these fish now exhibit an adfluvial life history, and it is unclear whether their descendents would re-express a fluvial life history. In the Missouri River basin, Big Hole River grayling theoretically provide a broader template for adaptation to novel environments or future environmental challenges because of their greater genetic diversity (Franklin and Frankham 1998; Lynch and Lande 1998). On the other hand, genetically depauperate populations can persist (e.g., Sunnyslope Canal). If maintaining diversity in captive populations of Big Hole grayling is important, then supplementation from the wild population is needed to limit genetic erosion. Adult grayling are presently at low abundance in the Big Hole River (MFWP 2005*a*, 2007), so genetic augmentation of the captive stocks awaits a demographic recovery of the Big Hole River population.

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