Genetic Status and Ancestry of Lake Populations of Upper Missouri River Arctic Grayling

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Introduction

Arctic grayling (grayling) were historically distributed widely but irregularly throughout the Upper Missouri River (UMR) above Great Falls, Montana (Vincent 1962). Their distribution has declined in the last century due to factors including habitat degradation, introduction of nonnative fish species, historic overharvest, and climate change (MAGW 2022). Currently, there are 19 extant populations of Arctic grayling within the UMR which includes aboriginal populations in several lakes and rivers in the Big Hole and Centennial valleys (CV), as well as introduced populations in 12 mountain lakes (USFWS 2020, MAGW 2022). At least six viable populations of grayling also exist in mountain lakes outside the UMR in western Montana and Wyoming.

Introduced mountain lake populations of grayling in Montana and Wyoming have significant conservation value because they were established with aboriginal UMR grayling (Peterson and Ardren 2009). Beginning in the 1890s, a propagation program was initiated using grayling from the CV (Henshall 1907, Beal 1951, BFH 1907, Kelly 1931) which eventually expanded to use fish from the Madison River by 1914 (Beal 1953, Kaya 1990). It was also probable that Big Hole River grayling were included in the stocking program at some point (Peterson and Ardren 2009), especially since the Big Hole River was geographically close to the state's historic broodstock in Georgetown Lake. Between 1898 and 1965, approximately 100 million UMR grayling were planted throughout the west including in at least 120 Montana lakes and streams. At one point, this resulted in approximately 30 self-sustaining populations of grayling in mountain lakes throughout Montana and Wyoming that were ostensibly founded with UMR grayling (Kaya 1990). In 2014, the United States Fish and Wildlife Service (USFWS) determined introduced lake populations within the UMR that were founded entirely with UMR grayling would be included in the UMR Distinct Population Segment (DPS) of grayling if the populations (1) occupy natural habitat, (2) reproduce naturally, and (3) are not part of the captive brood (genetic) reserve program (USFWS 2020).

The high conservation value of introduced grayling populations founded with UMR grayling warrants a consistent genetic monitoring program. The conservation strategy for all lake populations of UMR grayling is to monitor temporal genetic variation and maintain a stable or increasing genetic effective population size (MAGW 2022). Monitoring should evaluate trends in genetic variation over time and determine genetic ancestry of individual populations to aid in drainage-specific conservation actions (MAGW 2022). It is recommended that introduced populations be monitored every 8 years (two generations) unless there is some evidence of a decline or uncertain genetic status. In that case, more frequent monitoring is advised, especially if a management action is taken. If future declines in genetic variation are observed, embryos, fry, or fingerlings from source populations with appropriate genetic ancestry will be

used to bolster genetic variation (i.e., genetic rescue; Whiteley et al. 2016). All introduced grayling populations (including those located outside of the UMR) effectively provide redundancy for the genetic legacy of aboriginal UMR grayling and may be potential donors for reintroduction projects and to improve variation of extant populations if necessary.

Although introduced and aboriginal UMR grayling populations had been identified (Peterson and Ardren 2009), it was unclear to what degree they replicated and provided redundancy for extant populations in the Big Hole, Madison, and the CV. Conservation of drainage-specific genetic variation, and thus, the evolutionary legacy of the species is the cornerstone of the conservation strategy for UMR grayling (MAGW 2022). This will be achieved, in part, by increasing the geographic distribution and establishing new populations of UMR grayling. However, understanding the ancestry of potential donor populations is essential to achieving the intent of population establishment (e.g., conserving drainage-specific variation). This is especially important when low abundances or capture efficiencies in aboriginal populations precludes using them as donor sources. While in-place conservation programs and measures to address local threats are the primary focus of conserving aboriginal populations, establishing genetic reserves to replicate population-level genetic variation and support establishment of redundant conservation populations is a critical secondary strategy. Two genetic reserves were established with Big Hole River grayling in the 1990s at Axolotl and Green Hollow lakes (Leary 1991). Although riparian habitat and instream flow improvements have stabilized the grayling population in the Big Hole River, the Big Hole genetic reserves are still managed to conserve existing genetic variation and serve as donor sources for new conservation populations. However, introduced populations that best replicate the CV and Madison River populations had not been identified, and specific genetic reserves for those aboriginal populations were never established.

Handkerchief Lake, in northwest Montana, was chosen to establish a CV grayling genetic reserve outside of the CV (Figure 1). The lake is not within the grayling's native range, but contained a viable population of introduced grayling for decades until it was treated with rotenone in 2013 to remove hybridized cutthroat trout (Grisak and Marotz 2002). A goal of the project was to re-establish a population of Arctic grayling upon completion of the hybrid trout removal. The initial grayling reintroduction in Handkerchief Lake was attempted using gametes from wild spawned CV grayling. However, the number of offspring produced was not large enough to establish a viable population. A subsequent decline in the aboriginal CV grayling population necessitated identification of alternative sources of CV-origin grayling.

Other opportunities exist within the Red Rock River sub-basin to incorporate grayling introductions into non-native fish removal projects aimed at restoring westslope cutthroat trout (e.g., Selway Lake and Winslow Creek). Additionally, a genetic infusion project is planned for the headwaters of the Red Rock River to address decreasing genetic variation of the

aboriginal CV grayling population. These projects required identification of suitable sources of CV-origin grayling in existing mountain lake populations.

The primary conservation strategy for Madison River grayling is to establish at least two viable populations in the drainage through stocking of eggs or fish (MAGW 2022). This effort is expected to require a minimum of 1,000,000 eggs per year for the next 10 years. Although the Big Hole River genetic reserves may be used to meet demographic goals of these efforts, grayling from populations with a Madison River genetic ancestry should be used for reintroductions when possible (MAGW 2022, Kovach et al. 2022).

The goals of this assessment were to:

1) Complete long-term genetic monitoring for lake populations of UMR Arctic grayling within the DPS (USFWS 2020, MAGW 2022).
2) Complete long-term genetic monitoring for self-sustaining grayling populations outside of the UMR that have value as genetic reserves but are not explicitly considered conservation populations because they occur outside of the geographic boundary of the DPS (USFWS 2020).
3) Evaluate historic stocking records and current genetic data to identify appropriate donor sources for the creation of CV Genetic Reserves and new conservation populations within the Red Rock River sub-basin.
4) Evaluate historic stocking records and current genetic data to identify suitable donor sources for new conservation populations within the Madison River sub-basin of the UMR.

METHODS

Study Area

The study included aboriginal grayling populations in Big Hole lakes, as well as 18 alpine lakes with introduced grayling throughout Montana and Wyoming (Figure 1). Twelve introduced populations are within the DPS and six populations are outside the DPS.

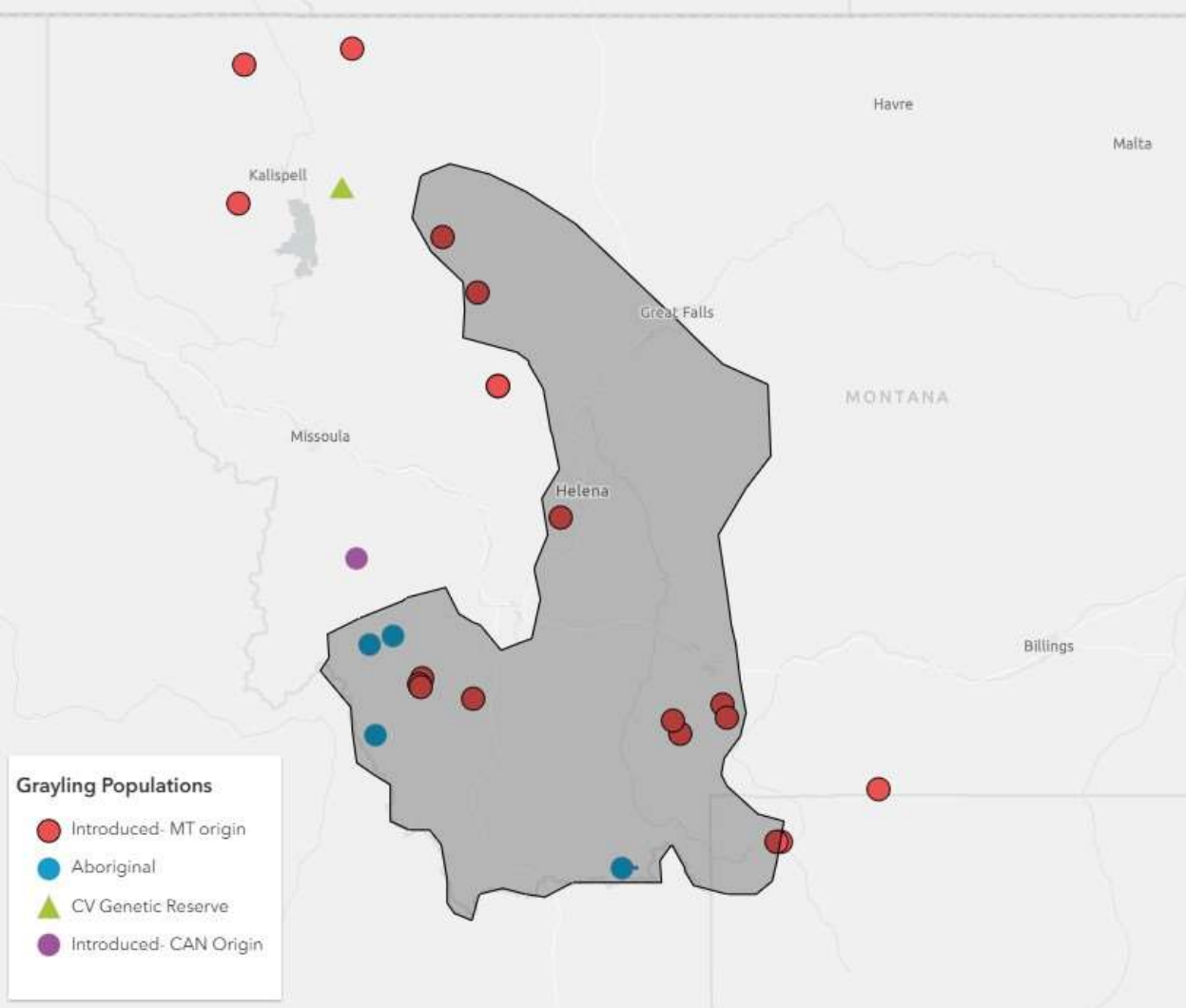

Figure 1. Self-sustaining populations of Arctic grayling in western Montana and Wyoming. The Missouri River upstream of Great Falls, MT represents the native range of Arctic Grayling and is shown in gray.

Fish sampling

We collected grayling tissue samples from all viable lake populations that hatchery records suggested were founded with UMR grayling.

Grayling genetic samples were collected from 2019 to 2021 using angling and/or gillnetting. We attempted to collect 30 samples from each lake. Tissue samples (0.25 cm^2) were collected from the pelvic fin and placed in 2 ml screw cap vials with 95% non-denatured ethanol. Relative population size was estimated as total abundance of spawning adults based on previous research, local professional opinion, mark/recapture estimates, or extrapolations of observed effective number of breeders to census sizes obtained in other grayling populations.

Goal 1: Long-term genetic monitoring for lake populations of UMR Arctic grayling within the DPS

Genetic Variation and Genetic Effective Population Size

We used two summary statistics to describe temporal patterns in genetic variation – mean expected heterozygosity across loci (H_e), and a rarefied measure of allelic richness (A_R). We used H_e and A_R because both indices are not influenced (i.e., biased) by sample size. Furthermore, H_e is directly and fundamentally a critical measure in population genetic theory, while A_R represents future evolutionary potential (Caballero and Garcia-Dorado 2013, Allendorf et al. 2014) and is sensitive to rapid demographic change making it ideal for genetic monitoring (Allendorf 1986, Luikart et al. 1998). Estimates of H_e and rarefied A_R were obtained using the 'hierfstat' package in Program R (Goudet 2004). Estimates of A_R are rarefied based on the smallest sample size analyzed by Kovach et al. (2021). Thus, A_R values can change based on which populations are included in the analysis (i.e., annual reports may show different A_r values). To make temporal comparisons, genetic stability was measured using a genic test for homogeneity (Kovach et al. 2021). This test examined whether allele frequencies were significantly different than random expectation between two samples. Last, we estimated the genetic effective population size (N_e) for each population where there was genetic data spanning at least 8 years. N_e is the summary statistic that governs the rate at which we expect genetic variation of a population to decline (Charlesworth 2009), and is central to Arctic grayling conservation efforts as a measure of genetic population viability (Jamieson and Allendorf 2012).

We compared the observed genotypic distributions at each microsatellite locus to expected random mating genotypic proportions (Hardy-Weinberg proportions) using *pegas* package (Paradis 2010) in Program R. We used these analyses to assess whether a sample appeared to be collected from a single random mating population, inbreeding, and population size. A deficit of observed heterozygotes can arise in a sample if it contains individuals from two or more genetically divergent populations, or the fish in it are experiencing a fair to high amount of inbreeding at the population level. Conversely, fish produced from a very small number of parents may show an excess of heterozygotes compared to expected random mating proportions (Pudovkin et al. 1996, 2010; Luikart and Cornuet 1999). Thus, for each population we summarized the number of microsatellite loci where we detected a significant ($P < 0.05$) excess or deficit of heterozygotes.

We used NeEstimator V.2 (Do et al. 2010) and the temporal method of Jorde and Ryman (2007) to estimate N_e. The temporal method estimates N_e based on observed changes in allele frequencies over time. The accuracy of this method strongly depends on length of time between sampling intervals, where longer periods result in higher accuracy because the signal

of genetic drift overwhelms sampling variation. When we had samples from multiple points in time (e.g., 2006, 2016, 2020), we estimated N_e for each temporal window, that is, 2006 to 2016, and 2006 to 2020 for the dates provided as an example. Alleles with a frequency of less than 0.02 (i.e., observed in only one fish) were not used to estimate N_e.

Last, we described the likelihood that each population will maintain existing genetic variation over multiple generations based on the observed trends in genetic stability, existing threats, and the relative population size.

Goal 2: Long-term genetic monitoring for self-sustaining grayling populations outside of the DPS

Genetic Variation

Estimates of genetic variation were calculated from six viable lake populations outside the DPS in an identical manner to lakes within the DPS (Goal 1). However, estimates of effective population size (N_e) and genetic stability over time could not be calculated because no comparable historic samples were available from any of the lakes.

Goal 3: Identify appropriate donor sources for the creation of CV Genetic Reserves and new conservation populations within the Red Rock River sub-basin

Stocking Records and Continued Existence of Introduced Grayling

We examined historic stocking and exportation records for grayling dating back to 1926 (MFWP database). Additionally, a literature review of historic documents and hatchery records was conducted and provided insight into the origins of grayling stocking programs prior to 1926 (BFH 1907, Henshall 1907, Beal 1951). Contemporary fish monitoring records were also examined to determine if lake populations existed without any record of stocking. State biologists responsible for the management of each lake were contacted for information regarding the status of self-sustaining grayling populations.

Private and diagnostic alleles

While hatchery records strongly suggest that grayling propagation was largely if not entirely sourced from the CV and the Madison River, we also assumed that grayling from the Big Hole River may have been used to found populations. Because we suspected populations may have been founded using a combination of aboriginal grayling sources, we considered 1) private and diagnostic alleles, 2) genetic differentiation, and 3) genetic assignment and admixture to determine primary ancestry. We focused our analyses on those alleles that were diagnostic for – unique to and characteristic of – the Big Hole and Madison populations of grayling or were private to the CV population of grayling. We defined diagnostic alleles as alleles that occurred

at a frequency of at least 0.20 in either the Big Hole or Madison but were completely absent or extremely rare (<0.002) in the CV, or alleles that were private to the CV and occurred at a frequency of >0.05. .

Genetic samples were available from all three potential source populations (Big Hole River, CV, and the Madison River), and new samples were collected from 17 lakes that were putatively founded from stocking Montana-origin grayling, 3 lakes with aboriginal grayling populations (Miner Lake, Mussigbrod Lake and Pintler Lake) and 2 streams from the CV (Long Creek and Odell Creek). Long Creek and Odell Creek were historically part of the larger CV grayling population that includes Red Rock Creek and were used as a "positive control" to verify that genetic data analysis can resolve whether populations are of CV origination. Similarly, Miner, Mussigbrod, and Pintler lakes are all in the Big Hole drainage and act as a "negative control" to verify that genetic data do not incorrectly identify non-CV origin fish as potentially of CV ancestry.

Multiple genetic samples were available for many of the grayling populations used in this analysis, but in order to minimize the effects of ongoing genetic drift (i.e., genetic divergence due to random allele frequency changes though time) we focused only on the oldest genetic sample from each location. All genetic data were produced using the same protocols and methods as described in Kovach et al. (2022).

Genetic differentiation

We used pair-wise estimates of F_{ST} (Weir and Cockerham 1984) to quantify genetic differentiation between grayling from CV and all other native and non-aboriginal populations of grayling. F_{ST} ranges from 0 to 1, where values from 0 to 0.05 are generally considered relatively low differentiation, values from 0.05 to 0.15 are moderate to high differentiation, and estimates > 0.15 generally suggest that populations are extremely different from one another.

Genetic assignment and admixture

The program STRUCTURE (Pritchard et al. 2000) was used to initially confirm that non-aboriginal populations appear to be primarily or entirely derived from grayling originating in the Big Hole River, CV Lake, and the Madison River. We tested a variety of scenarios that included subsets of the populations (e.g., we included/excluded other native populations of grayling), and subsets of the loci (e.g., we used all of the genetic data vs. a subset of the loci that best differentiated between the Madison, Big Hole, and Red Rock grayling populations).

We then coerced STRUCTURE to specifically estimate mean Red Rock, Big Hole, and Madison River ancestry within each of the native and non-aboriginal populations of grayling found throughout Montana. Specifically, we used multiple STRUCTURE runs (n = 10) with Red Rock, Big Hole, and Madison River as fixed population "sources", and tasked STRUCTURE with

estimating the mean population ancestry of all "unknown" populations (i.e., all other populations).

Overall genetic suitability criteria and ranking

We used a simple quantitative ranking method to identify the non-aboriginal populations that would be most/least suitable as "source-stocks" should there be a need to re-found aboriginal grayling. For each of the genetic analyses described above, we ranked populations from 0-4 based on the genetic results, where higher values represent a better "match" with native grayling from the CV. We then summed the values across all four categories (diagnostic alleles, private alleles, genetic differentiation, and genetic admixture). In other words, a population that appears to be a "perfect" match with the CV would have a value of 16, whereas a population that appears to be completely derived from another source (Madison, Big Hole, or otherwise) would have a value of 0.

We ranked populations according to the following criteria:

Category 1: Diagnostic alleles for Big Hole and Madison Rivers

0 – >5 diagnostic alleles
1 – 5 diagnostic alleles
2 – 3-4 diagnostic alleles
3 – 1-2 diagnostic alleles
4 – 0 diagnostic alleles

Category 2: Private alleles for CV grayling

0 – 0 private alleles
1 – 1 private alleles
2 – 2 private alleles
3 – 3 private alleles
4 – 4 private alleles

Category 3: Genetic differentiation from CV grayling

0 – $F_{ST} > 0.10$
1 – $F_{ST} < 0.10$ but > 0.075
2 – $F_{ST} < 0.075$ but > 0.05
3 – $F_{ST} < 0.05$ but > 0.025
4 – $F_{ST} < 0.025$

Category 4: Genetic assignment and admixture

0 – Mean CV ancestry < 0.25
1 – Mean CV ancestry > 0.25 but < 0.50
2 – Mean CV ancestry > 0.50 but < 0.75
3 – Mean CV ancestry > 0.75 but < 0.90
4 – Mean CV ancestry > 0.90

Goal 4: Identify appropriate donor sources for the creation of new conservation populations within Madison River sub-basin

The results from the analysis in Goal 3 can be viewed inversely to identify populations that appear to contain the most Madison River genetic ancestry and may be more suitable donors for conservation projects there. Therefore, introduced lake populations with the lowest scores from the four genetic categories described above were considered to be primarily of Madison River ancestry.

Results

Goal 1: Long-term genetic monitoring for lake populations of UMR Arctic grayling within the DPS

Genetic variation was calculated for 15 lake populations of grayling within the UMR. Temporal comparisons were only possible in eleven of the 15 lakes (Table 1). Estimates of N_e could only be obtained for the five populations in which multiple genetic samples have been collected over at least eight years.

Ten of the 11 populations with temporal data had contemporary values similar to the overall mean values and most exhibited little change in genetic variation over time. The genetic variation in the Pintler Lake population decreased and that population was determined to have a low ability to maintain extant variation over time. In four of the five populations where N_e was calculated, estimates suggested that populations were large and not at risk of significant losses of genetic variation over time. In Pintler Lake, the lower estimate of N_e (58) was consistent with the observed decline in genetic variation over time.

Goal 2: Genetic Status of Lake Populations of Grayling Outside the UMR Distinct Population Segment

The genetic status of viable lake populations of grayling outside the UMR DPS was measured between 2019 and 2021 (Table 2). Temporal comparisons were not possible from any of the lakes because historical samples either did not exist (i.e., Cascade, Cliff, Heart lakes), or were collected for protein electrophoresis and were not comparable (i.e., Elizabeth, Red Meadow, Rogers lakes). Therefore, only contemporary values of genetic variation were reported.

Table 1. Genetic status of self-sustaining lake populations of Arctic grayling within the Upper Missouri River Distinct Population Segment. Estimates of genetic variation include mean expected heterozygosity (H_e) and allelic richness (A_R). Estimates of A_R are rarefied based on the smallest sample size analyzed in Kovach et al. (2021). Thus, A_R values can change based on which populations are included in the analysis. Population size was estimated to be high (>1,000), moderate (1,000-100), or low (<100).

Lake Population	Origin	Population Size	**Ne (95% CI)** (Timeframe)	H_e	A_r	Genetic Stability	Ability to Maintain Extant Variation
Agnes	Introduced	High		0.83	9.46	Stable, P=0.182	High
Bobcat	Introduced	High	**204 (149-268)** (2006-2020)	0.79	6.88	Stable; P>0.069	High
Deer	Introduced	High		0.71	5.98	Stable; P=0.078	High
Emerald	Introduced	High		0.82	8.18	Stable; P=0.872	High
Gibson	Introduced	Low				N/A	Low
Grayling	Introduced	High	**478 (324-662)** (2012-2020)	0.74	5.56	Stable; P=0.585	High
Grebe	Introduced	High		None		N/A	High
Hyalite	Introduced	High		0.79	7.24	Variable (Increase); <0.001	High
Levale	Introduced	High		0.82	7.7	N/A	N/A
Odell	Introduced	High	**Infinity** (2006-2020)	0.80	9.18	Stable; P=0.839	High
Park	Introduced	High		0.81	8.16	Stable; P=0.12	High
Schwinegar	Introduced	High		0.81	7.77	N/A	N/A
Miner	Aboriginal	High	**208 (158-266)** (2006-2020)	0.81	9.26	Stable; P>0.19	High
Mussigbrod	Aboriginal	High	**4587 (3389-5965)** (2006-2020)	0.75	7.5	Stable (slight decrease); P=0.007-0.08	High
Pintler	Aboriginal	Mod	**58** (2009-2022)	0.67	4.63	Variable; P < 0.01	Low

Table 2. Genetic status of viable lake populations of Arctic grayling outside the Upper Missouri River Distinct Population Segment. Estimates of genetic variation include mean expected heterozygosity (H_e) and allelic richness (A_R). Estimates of A_R are rarefied based on the smallest sample size analyzed in Kovach et al. (2021). Thus, A_R values can change based on which populations are included in the analysis. Population size was estimated to be high (>1,000), moderate (1,000-100), or low (<100). No comparable historic samples were available from any of the lakes, therefore N_e and trend could not be evaluated.

Lake Population	N	Population Size	Year	H_e	A_R
Cascade	30	High	2021	0.82	7.88
Cliff	36	High	2020	0.79	7.76
Elizabeth	50	High	2019	0.82	8.92
Heart	24	High	2020	0.81	7.50
Red Meadow	31	High	2020	0.81	7.43
Rogers	50	High	2020	0.78	7.63

Goal 3: Identify appropriate donor sources for the creation of CV Genetic Reserves and new conservation populations within the Red Rock River sub-basin

Stocking Origin

A search of hatchery records identified that the origin of introduced grayling populations in Montana and Wyoming may have included CV, Madison River, and/or Big Hole River fish (Table 3). MFWP records indicated 138 lakes were stocked with grayling. Nineteen potential extant grayling populations were subsequently identified using monitoring reports and fish survey data. Ten populations were believed to contain fish originating from the CV or a combination of the Madison River and CV, six grayling populations had unknown origins, two were believed to be of Big Hole River origin, and one was known to contain grayling of Canadian origin.

Shared Big Hole and Madison diagnostic alleles (i.e., alleles found in the Big Hole and Madison but not CV) were found in all non-aboriginal populations; out of a total of seven possible diagnostic alleles, we detected between one and five diagnostic alleles in each non-aboriginal population (Table 4). This strongly suggests that all of the non-aboriginal populations have some Big Hole or Madison River ancestry. However, data from the single diagnostic allele for the Madison River and the single diagnostic allele for the Big Hole River provide evidence that the observed non-CV ancestry is from the Madison River rather than the Big Hole; the allele diagnostic for the Madison was found in almost all stocked populations, whereas the allele diagnostic for the Big Hole was not found in any of the stocked populations. As expected, Madison and Big Hole diagnostic alleles were not detected in grayling from Odell Creek and Long Creek (CV controls).

Among twelve-microsatellite loci, there was one diagnostic allele for the Big Hole River, one diagnostic allele for the Madison River, and five alleles that were diagnostic for both the Madison and Big Hole (i.e., the allele was at high frequency in at least one of those populations and also present in the other population but not present in the CV population; Table 5). Non-aboriginal populations that were entirely derived from the CV should have none of those alleles. Additionally, there were four private alleles found in CV grayling, but not the Madison River or the Big Hole River (Table 5). Those alleles should be present in populations that are derived primarily from the CV and absent in those that are primarily derived from the Madison or the Big Hole River. Private CV alleles from Red Rock Creek were found in nearly all of the non-aboriginal populations (Table 6), suggesting that all non-aboriginal populations likely have some CV ancestry. The population with the fewest private CV alleles was Grayling Lake (zero) and the populations with the most private CV alleles were Elizabeth Lake and Odell Lake (Big Hole) (all four private alleles were detected in both populations). As expected, all four private alleles were detected in Odell Creek, but only two of the Red Rock private alleles were detected in Long Creek. It is critical to note that the Long Creek sample was the smallest sample in this

analysis, which greatly reduced our power to detect all four private alleles, and thus, this result simply reflects sample constraints.

Table 3. Lacustrine grayling populations in Montana and Wyoming and their putative stocking source based on hatchery records and management reports (Gander 2019).

Population	Drainage	Putative Stocking Source Based on Hatchery Records	FWP Management Region
Agnes Lake	Big Hole	Madison/Centennial	3 Southwestern MT
Bobcat Lake	Big Hole	Centennial	3 Southwestern MT
Odell Lake	Big Hole	Centennial	3 Southwestern MT
Schwinegar Lake	Big Hole	Madison/Centennial	3 Southwestern MT
Deer Lake	Gallatin	Madison/Centennial	3 Southwestern MT
Emerald Lake	Gallatin	Madison/Centennial	3 Southwestern MT
Grayling Lake	Gallatin	Madison/Centennial	3 Southwestern MT
Hyalite Lake	Gallatin	Madison/Centennial	3 Southwestern MT
Gibson Reservoir	Sun	Big Hole	4 North Central MT
Lake Levale	Sun	Big Hole	4 North Central MT
Park Lake	Missouri	Madison/Centennial	3 Southwestern MT
Elizabeth Lake	Belly	Unknown	Glacier National Park
Fuse Lake	WF Rock	Canada	2 Western MT
Heart Lake	Blackfoot	Unknown	2 Western MT
Red Meadow Lake	Flathead	Unknown	1 Northwestern MT
Rogers Lake	Flathead	Unknown	1 Northwestern MT
Cliff Lake	Clarks Fork	Unknown	5 South Central MT
Meadow Lake	Green River	Unknown	Wyoming Fish and Game
Cascade Lake	Yellowstone River	Madison/Centennial	Yellowstone NP

Table 4. The total number of unique alleles diagnostic for the BH, MAD, and BH/MAD (see Table 1 above) that are found in each non-aboriginal (i.e., stocked) population of Arctic grayling. The populations with an asterisk (*) are native populations that act as controls; populations in the Big Hole River basin should share many unique diagnostic alleles with the Big Hole River population, and conversely, native populations in the Centennial Valley should have few or none of the unique alleles that are diagnostic for the Big Hole or the Madison.

Population	BH	MAD	BH/MAD	Total
Meadow Lake	0	0	4	4
Elizabeth Lake	0	1	3	4
Park Lake	0	1	3	4
Agnes Lake	0	1	4	5
Bobcat Lake	0	1	3	4
Odell Lake	0	1	3	4
Emerald Lake	0	1	3	4
Grayling Lake	0	1	2	3
Hyalite Lake	0	1	2	3
Deer Lake	0	1	2	3
Sunnyslope Canal	0	1	0	1
Odell Creek*	0	0	0	0
Long Creek*	0	0	0	0
Miner Lake*	1	0	4	5
Mussigbrod Lake*	1	0	3	4
Pintler Lake*	1	0	3	4
Cliff Lake	0	1	3	4
Heart Lake	0	1	4	5
LeVale Lake	0	1	4	5
Rogers Lake	0	1	4	5
Red Meadow Lake	0	1	4	5
Schwinegar Lake	0	1	2	3
Cascade Lake	0	1	4	5

Table 5. The number of unique alleles by locus and by population that are found in either or both of the Big Hole (BH) or Madison (MAD) and alleles that are private to the Centennial Valley (CV). BH is the Big Hole River, MAD is the Madison River, and BH/MAD are alleles with frequency > 0.20 (in at least one population) that are shared between the Big Hole River and Madison River, but are not found in, or are extremely rare (frequency < 0.005) in the Centennial Valley (CV). Private alleles for the CV occurred at a frequency of >0.05. Values with an * symbol denote a unique allele "spectrum", where a range of alleles at either the upper or lower end of the microsatellite allele distribution are unique to one of the focal populations.

Population	Frequency	Tar100	Tar104	Tar115	Tar105	Tar101	Tar103	Tar109	Tar114	Tar112	Tar106	Tar108	Tar110
Private BH	>0.20												1*
Private MAD	>0.20						1						
BH/MAD	>0.20					1		1	1	1	1		
Private CV	>0.05	1							1*			1*	1*

Table 6. The total number of private alleles from CV Arctic grayling that are found in each non-aboriginal (i.e., stocked) population of Arctic grayling. The populations with an asterisk (*) are native populations that act as controls demonstrating that Red Rock private alleles should be rare/non-existent in native populations from the Big Hole River basin, and reciprocally, those alleles should be present in other populations of grayling the Centennial Valley.

Population	RR
Meadow Lake	1
Elizabeth Lake	4
Park Lake	3
Deer Lake	2
Agnes Lake	3
Bobcat Lake	3
Odell Lake	4
Emerald Lake	3
Grayling Lake	0
Hyalite Lake	1
Sunnyslope Canal	1
Odell Creek*	4
Long Creek*	2
Miner Lake*	2
Mussigbrod Lake*	0
Pintler Lake*	0
Cliff Lake	3
Heart Lake	1
LeVale Lake	1
Rogers Lake	1
Red Meadow Lake	1
Schwinegar Lake	3
Cascade Lake	2

Genetic differentiation

Grayling from the CV are moderately to extremely different than most grayling populations in Montana or Wyoming (Table 7). The only exceptions were Elizabeth Lake, Agnes Lake, and Odell Lake, Cliff Lake and Schwinegar Lake all of which were similar to the CV.

As expected, Odell Creek and Long Creek were similar to the CV, whereas Miner Lake, Pintler Lake and Mussigbrod Lake were all quite divergent from CV grayling. Importantly, our genetic sample from the Madison River was quite different from our sample of grayling from the CV (F_{ST} = 0.179). Strong differentiation between the Madison River and the CV increases our power to estimate proportional ancestry in hybridized populations of grayling founded from the Madison River and the CV (section below).

Table 7. Pair-wise estimates of genetic differentiation (F_{ST}) between grayling from the CV and all other non-aboriginal (italics) and native populations of Arctic grayling (bold).

Population	Waterbody Location by Drainage	F_{ST}
Big Hole River	**Big Hole River**	**0.077**
Madison River	**Madison River**	**0.179**
Meadow Lake	*Green River (WY)*	*0.115*
Elizabeth Lake	*Belly River*	*0.027*
Park Lake	*Upper Missouri River*	*0.058*
Deer Lake	*Gallatin River*	*0.117*
Agnes Lake	*Big Hole River*	*0.032*
Bobcat Lake	*Big Hole River*	*0.063*
Odell Lake	*Big Hole River*	*0.017*
Emerald Lake	*Gallatin River*	*0.068*
Grayling Lake	*Gallatin River*	*0.089*
Hyalite Lake	*Gallatin River*	*0.092*
Cliff Lake	*Clarks Fork River*	*0.044*
Heart Lake	*Blackfoot River*	*0.067*
LeVale Lake	*Sun River*	*0.068*
Rogers Lake	*Flathead River*	*0.069*
Red Meadow Lake	*Flathead River*	*0.069*
Schwinegar Lake	*Big Hole River*	*0.039*
Cascade Lake	*Yellowstone River*	*0.067*
Mussigbrod Lake	**Big Hole River**	**0.193**
Pintler lake	**Big Hole River**	**0.193**
Lower Miner Lake	**Big Hole River**	**0.176**
Long Creek	**Red Rock River**	**0.035**
Odell Creek	**Red Rock River**	**0.012**

Genetic assignment and admixture

Genetic assignment results consistently matched our *a priori* expectation, based on hatchery records, that grayling from the Madison, Big Hole and Red Rock form relatively unique populations, and that all non-aboriginal populations appear to be largely derived from either or both of the Madison River and the CV (Table 8).

Non-aboriginal populations appear to be mostly comprised of Madison River ancestry, followed by the CV, with relatively little Big Hole genetic contribution (Table 9). Among non-aboriginal populations, the proportion of CV ancestry varied from 0.044 to 0.713. Importantly, the data strongly suggest that all non-aboriginal populations contain ancestry from at least two if not all three potential donor populations (i.e., the populations appears to be a genetic mixture of the potential donor populations).

Table 8. Mean proportion CV ancestry for native and non-aboriginal (italics) populations of Arctic grayling that were potentially founded by CV-origin fish. A population that was entirely founded by CV-origin fish should have a mean ancestry value near 1.0, whereas a population that was founded from one of the other principle donors to hatchery operations (Madison River and Big Hole River) should have a mean ancestry value near 0.0. Values between 0.05 and 0.95 indicates the population is of hybrid origin.

Population	Big Hole	CV	Madison
Meadow Lake	*0.023*	*0.079*	*0.900*
Elizabeth Lake	*0.017*	*0.779*	*0.208*
Park Lake	*0.015*	*0.916*	*0.075*
Deer Lake	*0.007*	*0.021*	*0.972*
Agnes Lake	*0.027*	*0.796*	*0.179*
Bobcat Lake	*0.016*	*0.925*	*0.059*
Odell Lake	*0.015*	*0.795*	*0.193*
Emerald Lake	*0.013*	*0.698*	*0.299*
Grayling Lake	*0.008*	*0.148*	*0.842*
Hyalite Lake	*0.013*	*0.868*	*0.119*
Sunnyslope Canal	*0.006*	*0.052*	*0.939*
Cliff Lake	*0.008*	*0.547*	*0.451*
Heart Lake	*0.026*	*0.188*	*0.783*
LeVale Lake	*0.032*	*0.127*	*0.832*
Rogers Lake	*0.019*	*0.115*	*0.864*
Red Meadow Lake	*0.013*	*0.096*	*0.887*
Schwinegar Lake	*0.014*	*0.924*	*0.063*
Cascade Lake	*0.012*	*0.682*	*0.306*
Lower Miner Lake	0.987	0.007	0.007
Mussigbrod Lake	0.989	0.006	0.006
Pintler Lake	0.916	0.014	0.074
Long Creek	0.007	0.981	0.015
Odell Creek	0.006	0.983	0.012

Overall genetic suitability criteria and ranking

Based on the four criterian analyzed in this study, five introduced populations emerged as being the best overall match with the CV (Table 9; Odell, Elizabeth, Schwinegar, Park and Bobcat lakes). It should be noted that there were no populations that were a “perfect” match for either the CV or Madison River because multiple lines of evidence suggest that all populations appear to have some ancestry from both.

Importantly, results from the positive (CV) and negative (Big Hole River) control populations suggest that, when combined, the genetic results described above successfully differentiated populations of CV-origin from “non-CV” origin; the two populations with best match to the CV population were Long Creek and Odell Creek, and the three populations that were the worst match were Mussigbrod, Pintler, and Lower Miner lakes.

Table 9. Final classifications for the four primary criteria used to identify and/or exclude potential donor populations that would be used to re-found CV Lake or a replicate of CV Lake (e.g., Handkerchief Lake). Each criteria (Criteria 1 = diagnostic alleles, Criteria 2 = private alleles, Criteria 3 = genetic differentiation, and Criteria 4 = admixture) ranges from 0-4, with 4 being populations that are most suitable and 0 being the least suitable. Populations with the highest scores are most appropriate for translocation. Bold populations are native populations found in the Centennial and Big Hole River valleys and were used as positive and negative controls (respectively).

Population	Criteria 1	Criteria 2	Criteria 3	Criteria 4	Total
Meadow Lake	*2*	*1*	*0*	*0*	*3*
Elizabeth Lake	*2*	*4*	*3*	*3*	*12*
Park Lake	*2*	*3*	*2*	*4*	*11*
Agnes Lake	*1*	*3*	*3*	*3*	*10*
Bobcat Lake	*2*	*3*	*2*	*4*	*11*
Odell Lake	*2*	*4*	*4*	*3*	*13*
Emerald Lake	*2*	*3*	*2*	*2*	*9*
Grayling Lake	*2*	*0*	*1*	*0*	*3*
Hyalite Lake	*2*	*1*	*1*	*3*	*7*
Deer Lake	*2*	*2*	*0*	*0*	*4*
Sunnyslope Canal	*3*	*1*	*0*	*0*	*4*
Cliff Lake	*2*	*3*	*3*	*2*	*10*
Heart Lake	*1*	*1*	*2*	*0*	*4*
LeVale Lake	*1*	*1*	*2*	*0*	*4*
Rogers Lake	*1*	*1*	*2*	*0*	*4*
Red Meadow Lake	*1*	*1*	*2*	*0*	*4*
Schwinegar Lake	*2*	*3*	*3*	*4*	*12*
Cascade Lake	*1*	*2*	*2*	*2*	*7*
Lower Miner Lake	**1**	**2**	**0**	**0**	**3**
Mussigbrod Lake	**2**	**0**	**0**	**0**	**2**
Pintler Lake	**2**	**0**	**0**	**0**	**2**
Long Creek	**4**	**2**	**3**	**4**	**13**
Odell Creek	**4**	**4**	**4**	**4**	**16**

Goal 4: Identify appropriate donor sources for the creation of new conservation populations within Madison River sub-basin

The results of both the stocking records investigation and genetic ancestry analysis confirmed that all introduced populations of grayling in Montana and Wyoming were founded with a combination of CV and Madison River grayling. This is because multiple lines of evidence suggest that all populations appear to have some Madison River or CV ancestry. However, several populations appear to be primarily of Madison River origin, including Meadow Lake, Grayling Lake, Deer Lake, Sunnyslope Canal, Heart Lake, LeVale Lake, Rogers Lake, and Red Meadow Lake (Table 9).

DISCUSSION

Goal 1: Genetic Status of Lake Populations of Grayling within the UMR Distinct Population Segment

Genetic monitoring of self-sustaining lake populations of grayling within the UMR revealed a stable trend in genetic variation for most populations (Table 1). Ten of the 11 populations with comparable historic data showed a stable trend in genetic variation and were determined to have a high ability to maintain extant variation over time. The aboriginal population in Pintler Lake has declined demographically over time and genetic variation has been reduced as a result. This result has prompted a planned transfer of fish from Mussigbrod and Lower Miner lakes in 2025 to serve as a genetic rescue (Whiteley et al. 2016). Additionally, numerous beaver dams on the inlet stream may restrict spawning access during most years. This will be investigated in 2025 prior to the spawning run and any dams believed to be migration barriers will be notched to provide access. Genetic trends of all lake populations will be reassessed in 6-8 years.

Goal 2: Genetic Status of Lake Populations of Grayling outside the UMR Distinct Population Segment

Although no temporal comparisons were possible for the six viable lake populations outside of the DPS, contemporary values of H_e and A_r were similar to the mean values observed elsewhere. Moving forward, all six lakes are considered to have conservation value as potential donor populations for future restoration projects within the DPS. Genetic trends of all lake populations will be reassessed in 6-8 years.

Goal 3: Identify appropriate donor sources for the creation of CV Genetic Reserves and new conservation populations within the Red Rock River sub-basin

The genetic results presented above, especially the distribution of private alleles and STRUCTURE estimates of ancestry, appear to confirm hatchery records that indicate introduced populations of Arctic grayling in Montana lakes are composed of fish from the CV and the Madison River. There was fairly strong evidence that all introduced populations of grayling founded with UMR grayling likely have ancestry from both populations. Any effort to re-found the CV grayling population (either directly or via transfers from Handkerchief Lake) with existing non-aboriginal populations must accept that the future population would likely include genetic variation from the Madison River grayling population.

As such, the only means to conserve the wholly intact evolutionary legacy of the CV Arctic grayling is to conserve that population *in situ*. We recognize that this goal may not be achievable if the extant population of Arctic grayling in the CV does go extinct. The question then becomes, if re-founding the population is necessary, how problematic is ancestry from the Madison River? Theoretically, non-native genetic ancestry from the Madison River may be "maladapted" to conditions in the CV and Red Rock Creek (Taylor 1991). However, even without Madison River ancestry, non-aboriginal populations of grayling throughout Montana have been genetically isolated from the CV in novel habitats where they have likely undergone

some degree of local adaptation to their new environments. Thus, the presence of Madison River ancestry is, on some level, a moot point.

Furthermore, conserving an isolated non-hybridized population of Arctic grayling in Red Rock Creek is unlikely to represent historical conditions. The lack of genetic differentiation among known spawning areas of grayling in the Big Hole drainage (Peterson and Ardren 2009, Kovach et al. 2020) suggests that historically Arctic grayling may have been relatively homogenous at intermediate spatial scales (within major basins), at least compared to other salmonid fishes (Reilly et al. 2014). This might also suggest that natal site fidelity may be relatively poor, a pattern that has been confirmed in European grayling (Dalen 2016). Thus, rather than viewing Madison River genetic variation as wholly problematic and "unnatural", it is also plausible that the genetic infusion provided by Madison River ancestry could be beneficial (Tallmon et al. 2004, Whiteley et al. 2016) and more closely resemble historical patterns of gene flow that existed prior to European colonization of Montana.

While Madison River ancestry in all potential source populations is not ideal for creation of a specific CV genetic reserve, it is not an absolute deal breaker. With this in mind, it is important to note that a number of populations of non-aboriginal grayling appear to be largely, though not entirely, of CV-origin. When founding the Handkerchief Lake population, or augmenting the Red Rock Creek population itself, we strongly recommend that a minimum of three suitable donor populations are used to establish the new population. Fortunately, at least five populations appear relatively suitable (Table 10). Utilizing multiple donor populations will maximize the amount of CV genetic variation in the newly established population. Any one donor population almost certainly contains only a subset of the genetic variation found in Red Rock Creek, because they have been subject to two sources of stochastic genetic drift: (1) the initial genetic bottleneck that may have been imposed when collecting fish for the genetic brood itself and (2) subsequent genetic drift while isolated in their new habitats for ~25 generations.

This study identified alternate sources of CV-origin grayling for the establishment of a genetic reserve in Handkerchief Lake. As a result of this study, four of the five lakes with a primary genetic ancestry from the CV were chosen to supplement a CV genetic reserve in Handkerchief Lake and for additional conservation projects in the Red Rock River sub-basin. All lakes chosen as initial donors were located within the DPS. Elizabeth Lake is located in Glacier National Park approximately 15 km from a trailhead and is within the Saskatchewan River drainage and may be pursued in the future as an additional source of CV-origin fish (Table 10).

Table 10. Physical characteristics of surveyed grayling lakes most closely matched to Red Rock origin. The Red Rock score is out of 16 and the score is positively related to amount of Red Rock genetic origin (as described in Table 9). Species codes (GR- Arctic grayling, RB- rainbow trout, RBxWCT- rainbow/cutthroat trout hybrid, EB- brook trout).

Lake Name	Elevation (m)	Distance from Road (km)	Lake Size (ha)	Wilderness Area	Spawn Time	Spawn Location	GR Size (mm)	Other Species Present	CV Score
Odell	2,557	6.4	13.3	No	June/July	Outlet	200-320	RBxCT, EB	13
Elizabeth	1,492	12.8	78.9	No	Unknown	Inlet	Unknown	RB	12
Schwinegar	2,545	8.0	1.6	No	June/July	Inlet/Outlet	101-305	None	12
Park	1,926	0	12.9	No	May	Shoreline	152-305	WCT	11
Bobcat	2,562	6.4	2.4	No	June/July	Outlet	190-305	None	11

Goal 4: Identify appropriate donor sources for the creation of new conservation populations within Madison River sub-basin

Prior to this analysis, reintroduction efforts for grayling in the Madison River drainage utilized gametes of Big Hole River origin (i.e., Big Hole River genetic reserves in Axolotl and Green Hollow lakes). The results from this study may offer other opportunities for these efforts. Meadow, Deer, Grayling, LeVale, Rogers, and Red Meadow lakes were the closest genetic matches to historic Madison River grayling populations (Table 9). The most logistically feasible of these populations for propagation efforts are Rogers, Red Meadow, and Meadow lakes (WY), as all three are accessible by vehicle and currently support propagation programs for recreational grayling stocking (Table 11). As a result of this study, Rogers Lake became a donor source for projects to create conservation populations in the South Fork Madison River and Chiquita Lake in the Gallatin River sub-basin.

This study also provides donor options for the Madison River that more closely align physiographically with receiving waterbodies. Grayling spawn in the spring when water temperatures reach 10°C. This varies from early-May in mid-elevation rivers and lakes (1,200-1,800 m) to early-July in high elevation lakes (2,400-3,000 m). Previously, the elevation of donor lakes and receiving waterbodies have differed substantially such that egg introductions were logistically challenging. For example, eggs spawned and hardened from Big Hole genetic reserves (elevation: 1,750-2120 m) are typically eyed-up for introduction by late-May. However, at that time most high elevation tributaries of the Madison River are either not accessible due to remaining snowpack or water temperatures are too cold for successful incubation. This study may offer more appropriate Madison River donor populations with appropriate ancestry and spawning times.

Table 11. Physical characteristics of surveyed grayling lakes most closely matched to Madison River origin. The Madison score is out of 16 and the score is inversely related to the amount of Madison River genetic ancestry.

Lake Name	Drainage	Elevation (m)	Distance from Road (km)	Lake Size (ha)	Wilderness Area	Spawn Time	Spawn Location	GR Size (mm)	Other Species Present	Madison Score
Meadow	Green (WY)	2,407	0	20.2	No	June	Inlet	Unknown	None	3
Grayling	Gallatin	2,548	11.2	1.3	Yes	June/July	Unknown	208-296	None	3
Deer	Gallatin	2,780	10	6.4	Yes	June/July	Outlet	212-368	None	4
LeVale	Sun	2,242	40	4.9	Yes	Unknown	Unknown	100-150	None	4
Rogers	Flathead	1,219	0	96.7	No	May	Inlet	350-425	WCT	4
Red Meadow	Flathead	1,708	0	6.5	No	May/June	Inlet	178-279	WCT	4

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