High Genetic Variation Among and Within Arctic Grayling Populations in the Upper Missouri River Drainage, Montana

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Abstract

Data from 12 microsatellite loci were obtained from samples of Arctic grayling *Thymallus arcticus* from 15 water bodies in the upper Missouri River drainage, Montana. Of these, nine are believed to contain native populations and six introduced populations. Only slight (F_{ST}=0.003) allele frequency differences were detected among the samples from the 2007-2013 year classes produced in the Big Hole River. Levels of genetic variation were also temporally stable among the samples. Estimates of the effective number of breeders (N_b) showed an increasing trend among the Big Hole year class samples. The Big Hole River native population, therefore, does not appear to be at imminent threat of the adverse consequences of losing genetic diversity. It appears that descendants of Arctic grayling from the Big Hole River have successfully established an introduced population in the Ruby River. There are allele frequency differences between the Big Hole River and Ruby River samples (F_{ST}=0.030) and levels of genetic variation appear to be less in the Ruby River than the Big Hole River fish. Based on loss of heterozygosity, it appears the Ruby River had an effective founding population size of about 13. We suggest introducing additional fish to the Ruby River in an attempt to ameliorate the apparent founder effect. F_{ST}, STRUCTURE, and principal components analysis (PCA) all indicate substantial divergence among the samples. There appears to be less divergence among the introduced lake populations than the native ones. The introduced lake populations, based on stocking records, are believed to have been mainly established with fish from the Red Rock River drainage. F_{ST}, STRUCTURE, and PCA support this supposition. The relatively high divergence among the native populations also suggests that the stocking of many of these populations with large numbers of Red Rock fish has had no detectable homogenizing effect on the native populations.

Introduction

Arctic grayling *Thymallus arcticus* naturally occur in northern fresh and rarely brackish waters from Hudson Bay westward to the east slope of the Ural Mountains (Scott and Crossman 1973; Figure 1). In North America, two disjunct groups existed south of the general distribution of the fish. The disjunct group in Michigan has been extinct since the mid-1930’s (Scott and Crossman 1973). The other disjunct group, which still persists, historically was irregularly but, widespread in the upper Missouri River drainage (hence upper Missouri grayling) above the Great Falls in Montana and Wyoming (Kaya 1990).

The upper Missouri grayling possess two different life histories. Historically, the most common is believed to have been the fluvial life history in which fish spend their entire life in mainstem rivers and tributaries to the rivers. The adfluvial life form spends most of their life in lakes and uses inlet or outlet tributaries for spawning. Presently, the adfluvial life history predominates in upper Missouri grayling due to the loss of a number of fluvial populations and the establishment of Arctic grayling in a number of historically fishless lakes in the drainage. The fish used to stock these lakes are believed to have been mainly derived from the native Madison River fluvial and Red Rock Lakes adfluvial populations (Everett

1986). Whether or not these different life histories represent different evolutionary lineages has been a matter of debate.

Because of the extirpation of some populations and perceived declines in others, the fluvial Big Hole River population of upper Missouri grayling was petitioned for protection as an endangered species under the United States Endangered Species Act in 1991. At this time, there was some contention over whether the Big Hole River population warranted a Distinct Population Segment (DPS) classification, was part of a bigger upper Missouri grayling DPS, or did not warrant DPS recognition. Regardless, in 1994 the United States Fish and Wildlife Service without a formal policy in place for the designation of a DPS (USFWS 1994) determined that fluvial fish in the upper Missouri River drainage constituted a DPS. Their rational for this decision was that the fluvial fish in the upper Missouri River drainage were geographically isolated from other fluvial populations further north and were behaviorally distinct from adfluvial fish. They also determined that listing of the upper Missouri fluvial grayling was warranted but precluded by other higher priority listings (USFWS 1994). Thus, the Big Hole River, and apparently Madison River grayling, which were also considered to be fluvial (USFWS 1994), became candidates for listing.

A challenge to the USFWS contesting designation of upper Missouri fluvial grayling as warranted but precluded was filed with the U. S. District Court in Washington D.C. on May 31, 2003. As a result of this challenge, the USFWS performed a revision of their initial determination (USFWS 2007). This revision determined the upper Missouri fluvial grayling did not constitute a DPS. The fish, therefore, became an unlistable entity and was removed from the candidate list.

Shortly after publication of this revised finding, a complaint challenging it was filed with the Montana District Court in 2007. As a result of this challenge, the USFWS agreed to perform a new status review for upper Missouri grayling and subsequently submit a new finding. At this time, a formal policy for the designation of a DPS was in place (USFWS 1996). Using this policy, the new finding designated the presumed native upper Missouri grayling populations in the Big Hole River, Miner Lake, Mussigbrod Lake, Red Rock Lakes, and the Madison River-Ennis Reservoir as a DPS but, again found listing was warranted but precluded (USFWS 2010). The reversal to designate the presumed native upper Missouri grayling populations as a DPS was based on the decision that they were discrete since they were markedly separated geographically from other populations of Arctic grayling. Furthermore, they were considered significant since they occupied a unique ecological setting, loss of these populations would represent a gap in the species distribution, and allozyme (Everett 1986), mtDNA (Redenbach and Taylor 1999; Stamford and Taylor 2004), and microsatellite analyses (Peterson and Ardren 2009) all demonstrated that the upper Missouri grayling were genetically very different from those to the north in North America.

Again this finding was challenged. As a result the USFWS agreed to announce either a proposed rule or a finding of not warranted on or before September 30, 2014 (USFWS 2013). The latest decision found that the upper Missouri grayling did not warrant listing (USFWS 2014). Not surprisingly, this decision has been legally challenged (Earthjustice 2014).

From the above, it should be clear that the potential listing of upper Missouri grayling has been a long, probably arduous, and certainly a contentious process. It is not our intent in this report to enter into this process. Rather, we simply intend to extend the study of Peterson and Ardren (2009) describing the spatial and temporal patterns of genetic variation among and within presumed native and introduced populations of upper Missouri grayling. We hope the results will aid in making and implementing management and conservation decisions for the fish regardless of the present listing decision.

Samples and Methods

Samples were collected from 15 water bodies in the upper Missouri River drainage (Figure 2; Table 1). Most locations were sampled only once. Exceptions are the Big Hole River, Red Rock Creek, and the Ruby River. The Big Hole River and Ruby River fish comprised only young of the year fish based on size. There is basically little to no overlap in size between young of the year grayling in the Big Hole River drainage and older fish during the fall (Cayer and McCullough 2013; Figure 3). The remaining samples contained fish of various year classes. In the Big Hole River, samples were collected from multiple locations (Figure 2). Previous data and those of Peterson and Ardren (2009), however, indicated no evidence of spatial genetic structuring in the river or that the fish did not represent a single panmictic population. Samples from the Big Hole River collected during the same year but at different locations, therefore, were combined into a single sample.

Microsatellite analysis

Genetic analyses were conducted at the University of Montana Conservation Genetics Laboratory at the University of Montana, Missoula, Montana, USA. DNA was extracted using a detergent based cell lysis buffer and ammonium acetate protein precipitation followed by isopropyl alcohol DNA precipitation. DNA was re-suspended in 100ul TE buffer. DNA was diluted to approximately 20-100ng/ul and 12 microsatellite loci (Table 2) were polymerase chain reaction (PCR) amplified in a PTC-200 thermocycler (MJ Research Inc., Waltham, MA) using the QIAGEN Multiplex PCR Kit (QIAGEN, Valencia, CA). Multiplex reactions used a total volume of 10 ul and followed the QIAGEN microsatellite protocol. Two different PCR profiles were used; multiplex 1 and 3 used a touchdown profile with an initial annealing temperature of 63°C stepping down to 58°C, and multiplex 2 had an annealing temperature of 58°C. PCR products were visualized on an ABI3130xl Genetic Analyzer (Applied Biosystems Inc., Foster City, CA) in the Murdoch DNA Sequencing Facility at the University of Montana. Allele sizes were determined using the ABI GS600LIZ ladder, (Applied Biosystems Inc., Foster City, CA). Chromatogram output was viewed and analyzed using GeneMapper version 3.7 (Applied Biosystems Inc., Foster City, CA).

Temporal variation within streams

We used the log likelihood G test of Goudet et al. (1996) implemented in GENEPOP version 4.2 (Rousett 2008) to test for allele frequency differences among samples collected from the same stream in different years. Because multiple comparisons were made between or among samples, we accounted for the possibility that significant differences might arise simply by chance using the conservative modified level of significance proposed by Rice (1989). When there was evidence of genetic differences between or among samples at the modified level, we used the procedure of Weir and Cockerham (1984) available in GENEPOP version 4.2 to quantify the magnitude of genetic divergence between or among them by partitioning the total amount of genetic variation detected into that due to genetic differences between or among samples and genetic variation within samples (F_{ST}).

We had data available from seven consecutive year classes of Arctic grayling from the Big Hole River and three year classes from the Ruby River. Thus, we were able to estimate the effective number of breeders (N_b) in these samples using the program LDNe of Waples and Do (2008). This analysis is based on the amount of gametic phase disequilibrium (hence gametic disequilibrium) or the nonrandom association of alleles between pairs of loci in a sample. Related individuals will have a greater tendency to share alleles between loci than unrelated individuals resulting in increased gametic disequilibrium. Thus, as the number of parents producing a year class decreases, the proportion of related individuals in the year class and the amount of gametic disequilibrium in it are expected to increase. This will result in decreased values of N_b. It is possible, therefore, that N_b may decrease as sample size increases because individuals from more parents are included in the sample reducing gametic disequilibrium. We investigated this possibility in the Big Hole River samples by regressing N_b and the estimated proportion of half and full-siblings (estimated using ML-Relate, Kalinowski et al. 2006) on sample size.

We estimated levels of genetic variation in the year class samples using average expected heterozygosity (H_e) using GENALEX (Peakall and Smouse 2006) and allelic richness (A_R) using HP-Rare (Kalinowski 2005). A_R adjusts the average number of alleles detected per locus to the smallest sample size (in this case 17 from Long Creek, Table 1) so that the values are strictly comparable among samples.

Levels of genetic variation and patterns of genotypic and gametic variation within populations

We also estimated levels of genetic variation within populations using H_e and A_R. The Wilcoxon two-sample test was used to determine whether H_e or A_R significantly differed between groups of samples. We tested if observed genotypic distributions conformed to expected random mating proportions (Hardy-Weinberg) in the samples using the procedure of Guo and Thompson (1992) available in GENEPOP version 4.2 . We tested for the presence of gametic disequilibrium in the samples using the procedure of Weir (1996) also available in GENEPOP version 4.2. Because multiple comparisons for deviations from Hardy-Weinberg proportions were made within samples, we again accounted for the possibility that significant differences might arise simply by chance by using the modified level of significance. In both analyses, we used 1000 dememorizations, 100 batches, and 1000 iterations per batch.

Genetic divergence among populations

We used a variety of techniques to estimate amounts of genetic divergence among the population samples. First, we calculated F_{ST} between all possible pairs of samples. The Wilcoxon two-sample test was used to determine whether F_{ST} significantly differed between groups of samples. Next, we used the program STRUCTURE (Pritchard et al. 2000, 2007) to investigate if samples formed what appeared to be biologically sensible groups for likely values of a predetermined number of groups (K). In this analysis, we varied the number of prospective groups from one to 15. In these analyses we used a 'burn-in’ of 50,000 steps followed by 100,000 Monte Carlo Markov Chain analyses. We performed the analysis five times for each K and used the mean -ln probability of the five iterations to determine the most likely value of K. Finally, we subjected the allele frequency data to principle components analysis to determine if the samples fell into distinct, biologically sensible groups in multi-variate space.

Potential bottlenecks

When a population experiences a severe reduction in population size (bottleneck) within about five generations or less ago, it will have a tendency to possess an excess of heterozygotes at multiple loci compared to the amount expected if the population was in mutation drift-equilibrium (heterozygosity excess; Cornuet and Luikart 1997). Thus, we used the program BOTTLENECK of Piry et al. (1999) to determine if there was any evidence that some of the samples may have experienced a recent bottleneck. In these analyses, we used a two phased mutation model with ten percent variance and 90% single step mutations (SMM). The program calculates four statistical tests for evidence of a recent bottleneck. In the analyses, all tests qualitatively produced the same results. Thus, we report probability values only using the one-tailed Wilcoxon signed rank analysis for the presence of excess heterozygosity.

Results and Discussion

Hardy-Weinberg proportions and gametic disequilibrium

We examined whether or not our samples appeared to have come from panmictic mating populations by comparing observed genotypic to expected random mating (Hardy-Weinberg) proportions. There are five main reasons a sample may contain a deficit of heterozygotes compared to expected Hardy-Weinberg proportions. First, it may contain individuals from two or more genetically divergent populations (Wahlund effect). There may also be some inbreeding in the population (mating between related individuals in the population at a frequency greater than expected by chance). Next, some loci may possess null alleles. Some forms of natural selection can result in a deficit of heterozygotes compared to Hardy-Weinberg expectations. This selection, however, must be of particular forms and quite strong. Finally, nonrandom sampling can potentially result in a deficit of heterozygotes compared to random mating expectations. In reality, it will often be difficult to distinguish between a Wahlund effect and inbreeding because these possibilities are expected to result in a deficit of heterozygotes at multiple loci spread throughout the genome. In contrast, null alleles and selection will only influence genotypic proportions at the loci at which they exist or for selection at closely linked loci. Because the conditions under which selection will result in a deficit of heterozygotes are quite stringent, the presence of null alleles is often suspected when a locus or particular loci consistently demonstrate a deficit of heterozygotes among multiple samples.

Samples can also possess an excess of heterozygotes compared to Hardy-Weinberg expectations. This is mainly expected if they contain individuals from only a very small number of parents (e.g. Pudovkin et al. 1996, 2010; Luikart and Cornuet 1999; Allendorf et al. 2013). In this situation, observed genotypic proportions tend to be more similar to expected Mendelian than Hardy-Weinberg proportions. An excess of heterozygotes can also be present for statistical reasons. Low frequency alleles are often expected to show an excess of heterozygotes. For example, if a locus possesses only a single copy of an allele it must exist in a heterozygote. The expected value, however, will necessarily be less than one. With multiple low frequency alleles, therefore, a sample could contain a significant excess of heterozygotes for this reason alone.

Among the Big Hole River year classes, at the modified level of significance the 2007 (*Tar104*, P=0.0000; F_{is}=0.1402), 2008 (*Tar115*, P=0.0016; F_{is}=0.0591), 2009 (*Tar110*, P=0.0000; F_{is}=0.0304), and 2011 (*Tar106*, P=0.0000; F_{is}=0.0335) year classes all possessed one locus that showed a significant deficit of heterozygotes. Since the loci showing a deficit of heterozygotes differed among the samples, the deficits are unlikely due to the presence of null alleles.

Based on the study of Peterson and Ardren (2009) the Big Hole River appears to contain a single panmictic population and it is suspected there is little chance of migration from other populations into it. Thus, the deficits also do not seem likely to represent Wahlund effects. It is possible, however, that fish from some of the lakes in the drainage could possibly migrate into the river. We tested this using the migrant option available in GENECLASS2 (Piry et al. 2004). The dataset included all the Big Hole River samples treated as one and the Bobcat Lake, Miner Lake, Mussigbrod Lake, O'Dell Lake, and Pintler Lake samples treated separately. Of the Big Hole River fish sampled, only one was identified as potentially being a migrant having originated from O'Dell Lake. This apparently small amount of immigration into the Big Hole River is unlikely to result in a Wahlund effect.

This leaves inbreeding or the occurrence of family aggregations resulting in nonrandom sampling as the most likely explanations for the observed deficits of heterozygotes. The relatively low proportion of half and full-siblings we observed in the samples (Figure 4) suggests that nonrandom sampling is not likely the reason for the observation of a deficit of heterozygotes at some loci. Thus, inbreeding appears to be the most likely explanation.

The inbreeding explanation is somewhat supported by two observations. First, the N_b of the last two year classes which provided no evidence of a deficit of heterozygotes was substantially larger than that in the previous ones (Figure 5) where a deficit of heterozygotes was observed. The larger N_b of the 2012 and 2013 year classes is expected to result in a lower proportion of inbred matings and increase the likelihood of statistical conformity to

Hardy-Weinberg proportions. Next, when all the year classes are combined into a single Big Hole River sample, which should effectively result in a lower proportion of inbred matings because individuals from multiple year classes are included in it, no significant deviations from Hardy-Weinberg proportions were observed. This observation also suggests that if inbreeding is the cause for the deficit of heterozygotes in some year classes that it is not of sufficient magnitude and sporadic enough to constitute a genetic threat to the population's persistence since when year classes are combined the inbreeding effect on genotypic proportions appears to disappear. Thus, as a whole the population does not appear to be inbred.

Only the 2010 year class sample from the Ruby River demonstrated, at the modified level of significance, deviations from expected Hardy-Weinberg proportions. These deviations involved five loci (*Tar100*, P=0.0324, F_{is}=0.0217; *Tar104*, P=0.0127, F_{is}=-0.0868; *Tar105*, P=0.0022, F_{is}=0.0087; *Tar114*, P=0.0010, F_{is}=0.1387; *Tar106*, P=0.0000, F_{is}=0.0484). All the comparisons involved a deficit of heterozygotes except at *Tar104* where an excess was observed. Thus, there was no significant tendency ($X^2_1=1.800$, $P>0.40$) for the loci demonstrating significant deviations to have either an excess or deficit of heterozygotes. The biological meaning of these deviations, therefore, is unclear.

Of the population samples, only the ones from Miner Lake and the Ruby River indicated significant deviations of observed from expected Hardy-Weinberg genotypic proportions. In the Miner Lake sample, two loci (*Tar100*, P=0.0034, F_{is}=0.1310; *Tar114*, P=0.0199, F_{is}=0.0761) possessed a deficit of heterozygotes. It is unlikely, therefore, that these deficits are due to null alleles as the genotypic distributions at them generally statistically conformed to expected Hardy-Weinberg proportions in the other samples. Arctic grayling spawn in both the inlet and outlet streams of Miner Lake (Jim Olsen, MFWP, personal communication). The fish spawning in these streams could represent genetically divergent populations and samples from the lake could express a Wahlund effect. This hypothesis is directly testable by obtaining samples from the streams during the spawning season.

In the Ruby River sample, four loci demonstrated significant differences from expected Hardy-Weinberg proportions. Of these, three (*Tar114*, P=0.0033, F_{is}=0.00608; *Tar108*, P=0.0470, F_{is}=0.0368; *Tar110*, P=0.054, F_{is}=0.0032) showed a deficit of heterozygotes and one (*Tar106*, P=0.0018, F_{is}=-0.0009) an excess. In this sample, therefore, there was also no significant tendency ($X^2_1=1.000$, P=0.50) for the loci demonstrating significant deviations to have either an excess or deficit of heterozygotes. Again, the biological meaning of these deviations is unclear.

We also examined whether or not there was any evidence of gametic disequilibrium in the samples. There are five main factors that can result in gametic disequilibrium. First, a couple of loci examined can be on the same chromosome and relatively close to each other (linked loci). In this case, the pair of loci are not independently inherited and should demonstrate gametic disequilibrium among multiple samples. Second, the sample may contain individuals from only a few families or came from a population recently experiencing a significant founder effect or bottleneck. Third, the sample could possess a

Wahlund effect. The fourth reason is natural selection. Finally, hybridization, either intra or inter-specific, can result in gametic disequilibrium.

No pair of loci we examined demonstrated significant gametic disequilibrium among the majority of our samples. Thus, none of the loci appear to be closely linked.

Among the Big Hole River samples, all of them except the 2008, 2012 and 2013 year class samples contained significant gametic disequilibrium at four or more pairs of loci (Table 3). The reasons discussed for the observed deviations from expected Hardy-Weinberg genotypic proportions also pertain here and we suspect that this gametic disequilibrium is mainly the result of a small amount of inbreeding in some of the year classes. This small and sporadic amount of inbreeding, like deviations from expected Hardy-Weinberg genotypic proportions, appears to be detectable only at the year class level. When all the year class samples are combined there is no evidence of gametic disequilibrium in the resulting Big Hole River sample (Table 3). From a conservation perspective, therefore, we do not consider this potential small amount of inbreeding in some year classes to be a major concern.

All of the Ruby River year class samples contained many pairs of loci in gametic disequilibrium (Table 3). This probably is mainly the result of the population being established from a relatively small number of individuals. This gametic disequilibrium, mainly established by chance, can persist in the population for at least a few generations (Allendorf et al. 2013). Thus, we were not surprised to see that many loci in the combined Ruby River sample also demonstrated significant gametic disequilibrium (Table 3).

There was also significant gametic disequilibrium between many pairs of loci in the Red Rock River drainage sample (Table 3). This probably is partially due to the sample being a combination of fish collected from Red Rock Creek, O’Dell Creek, and Long Creek. Fish from these creeks exhibited slight allele frequency differences (Tables 4 and 5) and combining them into one sample probably has resulted in a Wahlund effect and the observed gametic disequilibrium.

Among the remaining samples, only those from Hyalite Reservoir and Deer Lake contained a few pairs of loci demonstrating significant gametic disequilibrium. The deviations observed in Hyalite Reservoir may be the result of immigration of fish out of the upstream genetically divergent population in Emerald Lake (Table 3). The reason(s) for the gametic disequilibrium observed in Deer Lake are not clear. This is definitely a closed system but, the lake contains inlet and outlet spawning fish. If these represent genetically divergent populations, samples obtained from the lake could contain a mixture of fish from the populations and the presence of a Wahlund effect could result in gametic disequilibrium in the sample.

In terms of population viability the presence of gametic disequilibrium is unclear unless it is due to prevalent inbreeding, very low N_e, or human induced gene flow resulting in outbreeding depression. With prevalent inbreeding or very low N_e, population viability is expected to be compromised because of inbreeding depression and the loss of genetic variation and not necessarily the gametic disequilibrium per se. Conversely, human induced gene flow resulting in outbreeding depression may result directly from the gametic disequilibrium if local adaptation involved co-adapted gene complexes. Considering upper Missouri grayling, only in the Ruby River where the observed gametic disequilibrium may be due to low $N_{e,}$ therefore, does the gametic disequilibrium appear to be indicative of the presence of a potential conservation issue. In the absence of or sporadic and very small levels of inbreeding, relatively robust N_e, and natural levels of gene flow the presence of gametic disequilibrium is most likely due to linkage or a Wahlund effect. In these situations, interpretation of the gametic disequilibrium from a conservation perspective is not straight forward. It could easily be neutral or even have positive effects on population viability.

Temporal variation and effective number of breeders among Big Hole River year classes

Among the Big Hole River year class samples, the allele frequencies significantly differed among them at nine loci (Table 7). These differences remained significant at the modified level providing good evidence that genetic differences existed among the year classes. These differences, however, accounted for only a trivial amount of the total genetic variation detected among the samples (Table 8). Thus, the allele frequencies in the Big Hole River population appear to have been relatively temporally stable over the last few years. Levels of genetic variation estimated as H_e and A_R also appeared to be high and temporally stable among the year classes (Table 1; Figures 6 and 7).

In populations maintained with a very low effective population size (N_e), one expects to observe an appreciable amount of genetic drift. In this situation, the allele frequencies in the population are expected to be temporally variable. In populations with very low N_e, levels of genetic variation, especially estimated as A_R, are also expected to decrease over time. The relative temporal stability of the allele frequencies and levels of genetic variation observed among the samples of the Big Hole River year classes, therefore, indirectly suggest that, at least recently, the population has been maintained by a fairly robust N_e. This is more directly supported by estimates of $N_{b.}$ From 2007-2011, N_b was generally in the neighborhood of 100^+ depending on whether all alleles at a frequency greater than 0.01, 0.02, or 0.05 were used in the analysis (Figure 5). In 2012, N_b increased about three fold compared to the values seen in 2011 (Figure 5). A further increase was observed in 2013 (Figure 5). Finally, there was no evidence that any of the year classes or that the population formed by combining the year class samples had experienced a fairly recent bottleneck. Based on these data, therefore, it appears from a genetics perspective that the short term evolutionary persistence of the Big Hole River population is not seriously compromised. This situation, however, could quickly change with detrimental environmental modification.

A caveat about the N_b estimates is that as the point estimate increases the precision of the estimate decreases (Table 7). Furthermore, as the number of low frequency alleles in the data increases the amount of gametic disequilibrium tends to decrease for statistical reasons resulting in higher N_b estimates (Figure 5, Table 9). Because of these statistical phenomena, therefore, we feel that it is most appropriate to interpret the estimates more qualitatively than quantitatively. Thus, the most important biological aspect of the estimates is the increasing trend and not the point estimates per se.

A concern we had about the N_b estimates is that they may be positively associated with sample size. This phenomenon could exist because as more individuals are sampled more unrelated fish could also be sampled. This would result in a lower amount of gametic disequilibrium and higher N_b estimates. We investigated this possibility in our data by regressing N_b on sample size. The regression was not significant (Figure 4) suggesting that the sampling scheme used and the numbers of fish collected each year did not detectably influence estimates of N_b.

The fairly robust estimates of N_b suggest that our samples contained a relatively small proportion of closely related individuals. Furthermore, the lack of an association between N_b and sample size suggests there should also be no association between the percentage of closely related individuals in a sample and sample size. We investigated these expectations by estimating the percentage of half and full-siblings in each sample and regressing the arcsine transformation of these values on sample size. As expected, the percentage of half-siblings (range 4.4 to 8.2 %; mean=5.9%) in the samples was relatively small and the percentage of full-siblings very small (range 0.1 to 0.8%; mean=0.53%). There was also no association between the estimated percentage of half or full-siblings in the samples and sample size (Figure 4).

The presence of half-siblings in all the samples indicates that at least some Arctic grayling have multiple mates within a year. We cannot determine from the data whether this pertains to males, females, or both sexes. Regardless, however, this does not appear to be an extremely uncommon situation as we observed about six percent of the fish in each year class to be half-siblings. Furthermore, the production of half-siblings does not seem to be an unusual characteristic of salmonid fishes (e.g. Garant et al. 2001; Seamons et al. 2004; Coleman and Jones 2011).

We cannot be certain whether the relatively large increase in N_b observed in 2012 and 2013 represents a fairly permanent increase or a temporary increase. Our hope, of course, is that it represents the former possibly indicating a positive response of the fish to numerous conservation measures that have occurred, and continue to occur, in the drainage. Arctic grayling usually do not reach sexual maturity in this drainage until age two (males) or three (females). This increase in N_b, therefore, could simply reflect unusually favorable environmental conditions in 2009-2011 resulting in unusually large year classes or 2012 and 2013 resulting in extremely high hatching success and/or juvenile survival.

Our results considering N_b are quite different than the apparent trend reported by USFWS (2010). They estimated "contemporary effective population size" using LDNe. This algorithm, however, was developed to estimate N_b for a single cohort. The USFWS samples contained individuals from multiple cohorts which will increase gametic disequilibrium when genetic differences exist among them as we have observed. It is unclear, therefore, what exactly the USFWS estimated. Because of this uncertainty, reliable interpretation of their data is precluded and should not be considered to be in conflict with ours.

We do not have reliable demographic data to relate to our genetic data. Arctic grayling occupy about 150 km of the upper Big Hole River and its tributaries (Emma Cayer, Montana Fish, Wildlife & Parks (MFWP), personal communication). This is a dynamic region and the conditions of particular sections can change significantly from one year to another. Arctic grayling also tend to be very mobile and congregate in certain areas and be exceptionally sparse in others. Establishment of permanent sampling sections and using conventional estimator or trend techniques, therefore, can provide misleading population estimates or trend data when extrapolated to the entire occupied area in the Big Hole River drainage. In the fall, collecting an adequate sample of young of the year grayling over a reasonable geographic area is not difficult and these results appear to produce reliable N_b estimates. Thus, MFWP has chosen to use N_b as a trend estimator for the Big Hole River population.

Founding population size, temporal genetic variation, and effective number of breeders among Ruby River year classes

In 1988, and 1990-1992, MFWP spawned Arctic grayling collected from the Big Hole River to establish a captive broodstock (Leary 1991; Jay Pravecek, MFWP, personal communication; Table 10). The primary purposes of establishing this broodstock were to have a source of fish for re-introduction efforts into the Big Hole River in case the fish in it became extinct and to provide a source of fish for re-introduction efforts of Arctic grayling in other waters. These fish have been maintained primarily in two small lakes; Axolotl Lake (beginning in 1989) which is on Bureau of Land Management property and Green Hollow (beginning in 1998) which is on private property. Initial attempts to raise them at the Bozeman Fish Technology Center and the Yellowstone River State Trout Hatchery resulted in failure. Thus, reliance was placed on the lakes in which the fish seem to perform well in terms of rearing and spawning. Progeny from additional Big Hole River fish were added to the Axolotl Lake broodstock from 2010-2013 (Jay Pravecek, MFWP, personal communication; Table 10). In 2010 and 2014, fish from Axolotl Lake were transferred to Green Hollow. These actions were taken to avoid the accumulation of significant genetic divergence between the fish in the two lakes due to genetic drift and possibly selection.

The Ruby River historically contained an Arctic grayling population (Byorth and Magee 1999). The native population, however, is believed to have become extinct sometime in the 1920’s probably due to the construction of Ruby Dam (Emma Cayer, MFWP, personal communication). In 1997, MFWP began a program to attempt to re-introduce Arctic grayling to the Ruby River using the Big Hole River broodstock. From 1997-2005, fish of age 0^+, 1^+, and 2^+ raised in captivity were introduced into the river with little or no success in terms of establishing a self sustaining population. These fish experienced a mortality rate of about 90% and very few were captured downstream in Ruby Reservoir (Emma Cayer, MFWP, personal communication). In 2003, the program began using stream side incubators and continued this practice until 2008. From 2009-2014, young of the year fish have annually been captured in the upper Ruby River indicating that some of the fish produced from the stream side incubators survived and successfully reproduced in the river as upper Missouri grayling in this region seldom reach the age of 5.

Among the three year class samples (2010-2012), which precludes any meaningful trend analysis, from the Ruby River significant allele frequency differences existed at eight loci. These differences remained significant at the modified level (Table 11). With the exception of the 2011 and 2012 year classes, the magnitude of these differences was generally larger than those observed among the Big Hole River year classes but, were still generally small (Tables 8 and 12). Overall, therefore, there was more divergence among the Ruby River year classes than the Big Hole River year classes (Tables 8 and 12). This suggests that, on the average, the Ruby River year classes had a somewhat smaller effective population size (N_e) than those in the Big Hole River generally resulting in larger temporal changes in allele frequencies. This is supported by estimates of N_b. The N_b using all alleles for the three year class samples we have from the Ruby River are substantially lower than those obtained from the same year classes in the Big Hole River: Ruby 2010=32.8; 2011=23.0; 2012=42.4.

Despite their common origin, there is good evidence that genetic differences exist between the Big Hole and Ruby River populations. When all the Big Hole River and Ruby River year class samples are combined into a single Big Hole River and a single Ruby River sample, significant allele frequency differences exist between them at all the loci analyzed (Table 13). These differences remain significant at the modified level but, are far from exceptionally large (F_{ST}=0.0302). Estimates of H_e (means; Big Hole 0.884, Ruby 0.829) and A_R (means; Big Hole 12.213, Ruby 10.077) also significantly differ (t_8=2.393, P<0.001) among the year classes collected from the two rivers. Thus, although the amount of allele frequency divergence between the populations is not large, on the average it appears that the Ruby River fish contain lower amounts of genetic variation than the Big Hole River fish especially when estimated as A_R.

The proportion of heterozygosity lost due to a founder effect assuming no subsequent genetic drift is expected to be $1/2N_e$ where N_e represents the effective population size of the number of founders and not that from the population from which the founders were obtained. The proportional difference in H_e between the Big Hole River and Ruby River fish is about 0.04 (Table 1). Thus, the N_e of the fish that established the Ruby River population appears to have been about 13. This may, however, be an underestimate as the Ruby River population was founded from the captive broodstock and not directly from Big Hole River fish. It is possible that the broodstock may have lost some genetic variation during its establishment and maintenance due to genetic drift and possibly selection compared to the Big Hole River population. The underestimate, if it exists, however, is expected to be slight given the fairly similar estimates of H_e in the Ruby River and Big Hole River samples.

In contrast to H_e, there was proportionately a larger decrease in A_R (16%) in the Ruby River compared to the Big Hole River samples (Table 1). This is somewhat to be expected. The loss of alleles is more sensitive to a reduction in population size than heterozygosity. Low frequency alleles that contribute little to heterozygosity tend to be lost during a bottleneck decreasing A_R before H_e (Allendorf 1986). In fact, over all the loci analyzed the combined Ruby River sample lacks 68 alleles at a frequency of 0.083 or less that were detected in the Big Hole River sample (Figure 9). Most of these undetected alleles (85%) exist at a frequency less than 0.05 in the Big Hole River sample. With the sample size of 81 from the Ruby River, it is unlikely (P=0.038) that alleles existing at a frequency of 0.02 or greater would not have been included in the sample because of sampling error. Thus, we conclude that many low frequency alleles in the Big Hole River population are absent from the Ruby River population resulting in lower A_R in the latter population.

Because of the smaller N_b, we expected to observe a higher proportion of closely related individuals in the Ruby River than Big Hole River year class samples. The data somewhat support this expectation. The proportion of half-siblings among the three Ruby River year classes ranged from 5.2 to 8.7% (mean=7.1%) and the proportion of full-siblings from 1.1 to 4.6% (mean=3.33%). The Wilcoxon two-sample test using the half-sibling data was not statistically significant between samples from the two rivers (t_8=0.394; P>0.20), but there was a significantly higher proportion of full-siblings (t_8=4.259; P<0.01) in the Ruby River than the Big Hole River samples.

Like the Big Hole River, the upper Ruby River represents very complex habitat. This again makes obtaining reliable population or trend estimates using conventional techniques extremely difficult if not impossible. Thus, MFWP has also now adopted the policy of estimating the trend in population abundance in the Ruby River by estimating N_b.

The smaller effective population size and apparently higher proportion of full-siblings in the Ruby River compared to the Big Hole River suggest that inbreeding and a reduction in amounts of genetic diversity should accumulate in the former faster than the latter. Furthermore, the apparent founding effective size is smaller and the loss of alleles is greater in the Ruby River population than MFWP ideally desires. Thus, there has been some discussion about attempting to ameliorate these conditions by attempting to introduce additional fish using the broodstock and stream side incubators. This action, however, would not be without risk. It is possible that the incubator fry may emerge and saturate the rearing habitat before those from natural reproduction emerge. This action, therefore, has the potential to suppress natural reproduction and in terms of founding effective size and number of alleles may have little or even a negative affect. We also strongly suggest that the genetic characteristics of the broodstocks be determined prior to any further introductions into the Ruby River. Because of genetic drift and potential adaptation to their environments it is possible the captive broodstocks may now be genetically quite divergent from the Big Hole River and Ruby River populations. If this is the case, then interbreeding between the Big Hole River broodstock and Ruby River fish could result in outbreeding depression. This should be considered a real concern as in European grayling *T. thymallus* local adaptations can be established over very short evolutionary time spans (Krogstad 2008).

In order to minimize these potential negative consequences of re-introductions, we suggest that if this action is implemented that only a small number of eggs produced from multiple females be placed in the incubators. Furthermore, we suggest the incubators be used about once every three years (about one generation) so that possible suppression of natural reproduction is at most sporadic. Regardless of what actions are taken, under good environmental conditions we do not expect that the N_e and N_b of the Ruby River fish will ever be equivalent or exceed that observed in the Big Hole River because of the reduced suitable habitat in the former. The fish are confined to about 60km of the upper Ruby River drainage (Emma Cayer, MFWP, personal communication).

Despite the apparent low founding N_e of the Ruby River population there is no significant indication that it or the individual year classes have experienced a recent bottleneck. There was not a significant heterozygosity excess in the 2010 (P=0.867), 2011 (P=0.117), and 2012 (P=0.212) year class samples or in the sample when all the year classes were combined (P=0.102). This suggests that a recent bottleneck has to be quite severe in order to be detected by the program BOTTLENECK and that negative results need to be viewed with caution.

Genetic divergence and levels of genetic variation among the Red Rock Lakes, O'Dell Creek, and Long Creek samples

Red Rock Lakes, which are completely on the Red Rock Lakes National Wildlife Refuge, contain a native population of Arctic grayling. These fish primarily occupy Upper Red Rock Lake and mainly spawn in its tributary Red Rock Creek. Some spawning also occurs in O'Dell Creek a tributary to Lower Red Rock Lake. The amount of spawning in the latter tributary is considered to be less than what occurs in Red Rock Creek and some fish are believed to inhabit the creek year round (Matt Jaeger, MFWP, personal communication).

Long Creek is a tributary to the Red Rock River about 25km downstream from the outlet of Lower Red Rock Lake. Arctic grayling, generally in what appears to be low abundance, are present in the lower reaches of the stream. The upper reaches of the stream are presently considered unsuitable habitat for Arctic grayling. Essentially no effort has been made to attempt to capture young of the year Arctic grayling from Long Creek (Matt Jaeger, MFWP, personal communication). Thus, it is unknown whether the creek contains a natural reproducing population, is maintained by emigration of fish out of potential upstream sources (Lima Reservoir, Upper Red Rock Lake, Lower Red Rock Lake, O'Dell Creek), or a combination of these processes.

Our samples from Red Rock Creek, O'Dell Creek, and Long Creek contained individuals from multiple year classes. We, therefore, could not obtain estimates of N_b for these samples.

Although the 2007 and 2008 Red Rock Creek, O'Dell Creek, and Long Creek samples all came from the Red Rock River drainage, significant allele frequency differences were detected among them at four loci (Table 4). These differences remained significant at the modified level indicating genetic differences existed among the samples. These differences, however, largely involved fish from the different creeks as pair wise comparisons indicated no significant allele frequency differences at the modified level were present between the two Red Rock Creek samples. Thus, the two Red Rock Creek samples were combined for further analyses.

Although allele frequency differences existed among the samples, the magnitude of these differences was quite small (Table 5). The differences between Red Rock and O'Dell Creek should not necessarily be interpreted to indicate that these samples came from genetically different spawning populations. Random assortment of fish from a single panmictic population into different spawning aggregates can result in genetic differences among the aggregates (Allendorf and Phelps 1981). A temporal sequence of allele frequencies will be necessary in order to attempt to genetically determine between these interpretations. It is also clear that among the samples Long Creek was by far the most divergent. This could indicate that it is a genetically different population than the fish in Red Rock Lakes or that it is mainly being maintained by a relatively small number of emigrants experiencing a founder effect, i.e. it is acting as a sink. We feel from a conservation perspective that it is important to determine whether or not Long Creek is mainly a sink. If so, the "population" in it is mainly being maintained by upstream sources and such sources should have conservation priority. In contrast, if the population is being maintained primarily by natural reproduction then it will have increased conservation value and it will be important to manage it as a genetically different population.

Estimates of H_e and A_R provide some indication that the Red Rock Creek N_e, is larger than that in O’Dell Creek and Long Creek. Of the three samples, Red Rock Creek has the highest values of these estimates and Long Creek the lowest (Figures 10 and 11). The greatest disparity among them exists for A_R again demonstrating that a reduction in the number of alleles is a more sensitive indicator of reduced genetic variation than heterozygosity. The values of A_R and H_e in Red Rock Creek and O’Dell Creek are still fairly robust suggesting over time that these populations have had fairly reasonable N_e and that levels of genetic variation within them are expected to diminish at a slow rate. Given the absence of an environmentally mediated catastrophic bottleneck, therefore, from a genetics perspective the Red Rock Creek and O’Dell Creek populations appear to have a relatively good probability of short term evolutionary persistence.

There was no indication that the samples obtained from the Red Rock River drainage came from populations that have experienced a recent severe bottleneck. There was not a significant ($P=0.604$) heterozygosity excess in the Red Rock Creek sample. Likewise, the Long Creek ($P=0.912$) and O’Dell Creek ($P=0.810$) samples did not contain excess heterozygosity. Finally, when all the samples from the drainage were combined into one there was still a lack of a heterozygosity excess ($P=0.661$).

Genetic divergence among upper Missouri populations

In order to reduce computational time in STRUCTURE, the Big Hole River population in this analysis contained ten randomly chosen individuals from each year class. The Red Rock Creek sample included 20 randomly chosen individuals from the 2007 and 2008 samples. The Ruby River sample contained all individuals in the 2010-2012 year class samples.

F_{ST} between the various grayling population samples was highly variable ranging from low to high levels of divergence (range 0.0053-0.2496). It generally, however, exceeded 0.05 (93%) and a large majority (70%) of the values exceeded 0.10 (Table 6). Usually, therefore, the different populations tended to show moderate to large amounts of genetic divergence based on F_{ST}. Notable exceptions involved the comparisons between Red Rock Creek, O’Dell Creek, and Long Creek, the Big Hole River and Ruby River, and Red Rock Creek and O’Dell Lake. The former two were expected. The Red Rock Creek, O’Dell Creek, and

Long Creek samples all represent fish from the Red Rock River drainage and with the exception of Long Creek to Red Rock Lakes gene flow between them is certainly possible. The Ruby River population was established using descendants of Big Hole River fish.

The similarity between the Red Rock Creek fish and those in O'Dell Lake in the Big Hole River at first seems counter intuitive. There is no stocking record for O'Dell Lake but, the population is believed to be introduced as about one million Arctic grayling were stocked in its outlet O'Dell Creek in 1928 (Lee Nelson, MFWP, personal communication). Arctic grayling stocking in Montana mainly involved fish from Red Rock Lakes (Everett 1986). Thus, it is tempting to postulate the similarity between O'Dell Lake and Red Rock Creek can be attributed to O'Dell Creek being stocked with fish originating from Red Rock Creek. There is a small amount of additional support for this speculation (data to be presented and discussed later) but, the probability this is simply a fortuitous similarity between the samples at a relatively small number of microsatellite loci cannot be casually dismissed.

In order to not bias values of F_{ST} between native populations downwards, we combined the two Red Rock Creek, O'Dell Creek, and Long Creek samples into a single Red Rock River drainage sample. For a given amount of gene flow, generally populations with a common, relatively recent origin are expected to show less divergence than populations with a more distant origin especially if the former have yet to reach equilibrium. Estimates of F_{ST} (Table 6) were significantly lower (t_{41}=4.570, P<0.001) between pairs of samples from introduced populations (range 0.0302-0.1606, mean=0.1006) than between pairs of native populations (range 0.0724-0.2390, mean=0.1608). Thus, on the average introduced populations tended to be genetically more similar, but certainly not identical, to each other than native populations. This is concordant with a common more recent origin of the introduced populations than the native populations.

We investigated the possibility that the introduced populations all may share at least a partial common origin with the Red Rock Lakes drainage fish by comparing the amount of genetic divergence observed between them and the Red Rock Lakes drainage sample and between them and the other native population samples. There was significantly less divergence (t_{33}=4.157, P<0.001) between the introduced populations and the Red Rock Lakes drainage sample (range 0.0297-0.1269, mean=0.0845) than the other introduced populations (range 0.0214-0.2356, mean=0.1412). On the average, therefore, the introduced populations tended to be less divergent, as estimated by F_{ST}, from the Red Rock Lakes drainage fish than the other native Arctic grayling. Similar results were obtained using allozymes by Everett (1986) and microsatellites by Peterson and Ardren (2009). The available F_{ST} data, therefore, lend some support to the presumption that many of the introduced populations were successfully stocked with fish from the Red Rock River drainage.

In our experience, the results we obtained from STRUCTURE were quite unusual. The -ln probability gradually increased from K=1-10, then it dramatically decreased at K=11 and 12, and finally increased to values fairly similar to those seen at K=10 when K was set at 13-15 (Figure 12). This makes determining the most likely value of K extremely arbitrary. Using the ΔK method of Evanno at al. (2005), one would choose K=13 as the most likely number of groups. The probability that K=13, however, is only slightly higher than many others (Figure 12).

When K was large, the clusters identified by STRUCTURE largely corresponded to our population samples. For example, when K=13 eleven of the individual population samples were identified as distinct clusters (Figure 13). The Grayling Lake and Deer Lake samples and the Long Creek and O'Dell Creek samples from the Red Rock River drainage formed the other two well defined clusters. This tendency for there to be high concordance between our population samples and the clusters determined by STRUCTURE at high K suggests that most of the population samples are genetically quite different from each other since the algorithm can potentially place individuals into any cluster regardless of population of origin.

We examined the results of K=2 because we were interested in whether or not this would result in a contrast between the native and introduced populations. This, however, was not the case (Figure 14). Basically the results contrasted what are suspected to be native populations in the Big Hole River drainage (Big Hole River, Mussigbrod Lake, Miner Lake, and Pintler Lake) and the introduced Ruby River population to the Red Rock River and Gallatin River native fish and the introduced lake populations. The native Madison River fish appeared to be intermediate to these two groups. Although this was not identified as a very likely outcome (Figure 12) with the exception of the Gallatin River population it makes some biological sense. The introduced lake populations, as presumed, appear to be mainly derived from Red Rock River drainage fish and the native populations are grouped by major river drainages; Big Hole, Madison, and Red Rock/Gallatin.

Results of the principal components analysis were very similar to those obtained by analysis of F_{ST} and STRUCTURE. The samples occupied a broad area in multivariate space especially along the first axis which accounted for 50% of the total variation but, some were placed quite close to each other (Figure 15). Overall, therefore, there was substantial genetic divergence among the samples but, some showed little divergence. The first axis was mainly a contrast of Big Hole River drainage and Red Rock River drainage fish. The Big Hole River drainage and its derivative the Ruby River tended to have positive values on this axis and the native Red Rock River fish negative values. The introduced lake populations had negative values and generally were more similar to Red Rock drainage than Big Hole drainage fish on this axis. The Madison River fish grouped with the Big Hole fish and the Gallatin fish grouped with those from the Red Rock drainage. The introduced populations formed a tighter group of samples than the native ones on this axis indicating less divergence among the introduced than native populations. These results, therefore, add support to the supposition that the introduced lake populations all share at least a partial common origin with fish originating from the Red Rock River drainage. Furthermore, they indicate substantial divergence among the native populations with the Madison fish being most similar to, but still quite divergent, from the Big Hole fish and the Gallatin fish being most similar to, but still quite divergent, from the Red Rock fish.

Populations whose genetic characteristics have been significantly influenced by organisms from a common source are expected to demonstrate reduced genetic divergence as they genetically converge towards the source. This phenomenon is not apparent among the samples from the native populations in the principal components analysis. Thus, it appears that the stocking of Arctic grayling into these waters has had little, if any, influence on their genetic characteristics. This is in contrast to what is generally observed in European grayling. In this species, stocking of fish into waters containing natural populations often results in significant genetic changes in the native populations (e.g. Koskinen et al. 2002; Susnik et al. 2004; Duftner et a. 2005; Gum et al. 2006) and is a major conservation concern in some areas. In contrast, given the present situation in the upper Missouri River drainage where the introduced populations mainly exist in isolated head water lakes, past stocking appears to have had little genetic impact on the native populations, and present stocking is basically confined to waters not containing Arctic grayling interbreeding between native and introduced fish does not appear to be a significant conservation issue for upper Missouri grayling.

Genetic variation within upper Missouri populations

Levels of genetic variation estimated as H_e and A_R were highly variable among the samples. H_e was generally high in the samples and ranged from 0.883 to 0.657 (Figure 10). A_R ranged from 11.440 to 4.820 (Figure 11). Often when populations are established from another they exhibit reduced amounts of genetic variation compared to the source population because of founder effects and possibly subsequent genetic drift (e.g. Nakajima et al. 1991; Leary and Allendorf 1993). Thus, we expected if there was a difference in levels of genetic variation between the introduced and the native Red Rock River drainage fish that it would be lower in the former samples. Excluding the Ruby River sample which is derived from Big Hole River fish, there was, however, no statistically significant difference between the mean H_e of the introduced populations (range=0.808-0.697, mean=0.763) and that observed among the Red Rock River drainage samples (range=0.766-0.667, mean=0.721, t_6=0.989, P>0.40). There was also, no statistically significant difference between the mean A_R of the introduced populations (range=9.17-7.16, mean=7.29) and that observed among the Red Rock River drainage samples (range=9.34-5.26, mean=8.02, t_6=0.424, P>0.40). Thus, with the exception of allele frequency divergence there was no detectable evidence that, on the average, the introduced populations had experienced founder effects and subsequent genetic drift significant enough to significantly reduce levels of genetic variation.

In general, our samples tended to contain appreciable amounts of genetic diversity. This and the lack of evidence for appreciable founder effects and genetic drift in most of the introduced populations indirectly suggest that the majority of them have been maintained by a fairly large N_e and have not experienced recent bottlenecks of substantial magnitude. We more directly tested whether or not our samples came from populations that had experienced recent bottlenecks using the program BOTTLENECK. Based on this analysis only the Bobcat Lake sample showed evidence of having come from a population that had undergone a recent bottleneck (P=0.0170). Despite it apparently having undergone a fairly recent bottleneck, the Bobcat Lake population still appears to contain a fair amount of genetic diversity (Figures 10 and 11).

The BOTTLENECK results need to be interpreted with some caution. First, the power of the test was statistically weak as we had data from only 12 loci. Furthermore, the analysis will detect bottlenecks that occurred at best four to five generations ago and as the severity of the bottleneck decreases the length of time it remains detectable also decreases. Thus, some populations could have undergone fairly recent bottlenecks, and this is suggested by other evidence (e.g. Ruby River and Long Creek) but, the bottlenecks occurred to far in the past or were not severe enough to be detectable with the BOTTLENECK algorithm. Regardless, most samples suggest that the populations contain appreciable amounts of genetic diversity and, therefore, low levels of genetic diversity presently do not appear to represent a genetic threat to their persistence.

Conclusions

Allele frequencies and levels of genetic variation in samples from seven consecutive year classes of Arctic grayling from the Big Hole River were temporally stable. Levels of genetic variation in the samples were also high. Estimates of N_b increased dramatically in 2012 and 2013 compared to the previous five years. Based on deviations from expected Hardy-Weinberg genotypic proportions and the presence of gametic disequilibrium, there appeared to be a small amount of inbreeding in some of the year classes. There appears to be very little inbreeding at the population level, however, as evidence for it is not present when year class samples are combined. Finally, there was no evidence that the population has experienced a recent severe bottleneck. The Big Hole River population, therefore, does not appear to be in imminent jeopardy of experiencing the adverse effects of inbreeding and the loss of genetic diversity.

A population in the Ruby River has apparently been established from descendants of fish spawned from the Big Hole River. Based on estimates of effective founding population size, N_b, reduced A_R, and the presence of gametic disequilibrium in samples from the 2010-2012 Ruby River year classes the Ruby River population appears to have experienced a founder effect. In order to ameliorate the founder effect, we suggest that attempts be made to introduce additional fish to the Ruby River but, caution needs to be exercised in these attempts. The possibility of outbreeding depression and the suppression of natural reproduction should be considered when contemplating potential additional introductions.

Significant allele frequency differences exist among the Red Rock Creek, O’Dell Creek, and Long Creek samples from the Red Rock River drainage. Red Rock Creek and O’Dell Creek, both tributaries to Red Rock Lakes, therefore, may contain genetically divergent spawning populations inhabiting Red Rock Lakes. Alternatively, these differences could simply result from the random assortment of fish from the lakes into these tributaries (Allendorf and Phelps 1981). The magnitude of genetic divergence between these two potential populations, however, was relatively small and there is certainly the possibility for gene flow between them. Both samples appear to have reasonably high levels of genetic variation and neither appears to have come from a population that experienced a recent bottleneck. Thus, both samples appear to have come from a population that has been maintained by a relatively high enough N_e over time to prevent a significant loss of genetic variation. The gametic disequilibrium observed in the sample formed by combining the two is probably simply the result of combining genetically different samples.

It is unknown whether the Arctic grayling in Long Creek are mainly the result of natural reproduction in the creek or represent immigrants from upstream populations. If the latter, then its continued existence would largely depend on the persistence of the upstream fish and conservation efforts should be focused on them. Conversely, if the fish in Long Creek represent a self sustaining population, then their conservation value would greatly increase. Thus, we feel that determination of the source of fish in Long Creek should be considered a high conservation priority.

Based on F_{ST} there was a highly variable range of genetic divergence between pairs of populations. On the average, there appeared to be less divergence between introduced than native populations probably reflecting a more recent common origin and less divergence time among the former populations. The introduced lake populations are believed to have mainly been established with fish derived from Red Rock Lakes. F_{ST} values somewhat support this belief as introduced populations on the average tend to be genetically more similar to the Red Rock River drainage sample than any other presumed native population.

Results of the principle components analysis yielded very similar results to analysis of F_{ST}. In addition, the principle components analysis suggested that the past stocking of Arctic grayling into waters containing native populations generally had no detectable genetic influence on the native populations. The introduced lake populations appeared to be less divergent than the native populations and tended to be genetically more similar to the fish in the Red Rock and Gallatin drainages than those in the Big Hole and Madison drainages. These data also support the supposition that the introduced lake populations all share at least a partial origin from Red Rock fish.

STRUCTURE results indicated that most samples tended to represent a relatively distinct group of fish. In terms of native populations and K=2, the analysis contrasted native Big Hole River and Red Rock River drainage fish. The Gallatin River drainage native fish and those from introduced lake populations clustered with those from the Red Rock River drainage again supporting the presumption that these introduced populations were largely established from Red Rock fish. The native Madison River fish were intermediate to the Big Hole and Red Rock clusters. F_{ST} and principal components also indicated that there was more similarity between the Red Rock and Gallatin native fish and the Big Hole and Madison native fish than between fish from the groups.

Based on conformity of observed to expected Hardy-Weinberg genotypic proportions and the lack of gametic disequilibrium, most samples appear to have come from panmictic populations. The exceptions are Ruby River, Miner Lake, Deer Lake, and Hyalite Reservoir. In the Miner Lake sample, we observed a significant deficit of heterozygotes compared to Hardy-Weinberg expectations. Significant gametic disequilibrium was observed in the Deer Lake sample. Arctic grayling spawn in the inlet and outlet creeks to both lakes. Genetic differences between the inlet and outlet spawning groups in the lakes could result in the presence of a Wahlund effect in samples obtained from the lakes and account for the observed results. The gametic disequilibrium observed in the Hyalite reservoir sample may be due to immigrants out of upstream Emerald Lake. The deviations from Hardy-Weinberg expectations in the Ruby River sample involved both an excess and deficit of heterozygotes.

The biological meaning of these deviations, therefore, is unclear. The gametic disequilibrium observed in the Ruby River sample is probably the result of its relatively low effective founding size.

Levels of genetic variation estimated as H_e and A_R were highly variable among the samples but, were generally appreciable. This suggests that most, if not all, the populations have been maintained by a relatively substantial N_e. With the exception of Bobcat Lake, there was also no evidence that any of the populations had undergone a recent severe bottleneck. Since most populations appear to contain appreciable amounts of genetic diversity, low levels of genetic diversity presently do not appear to represent a genetic threat to their persistence.

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Literature Cited

Allendorf, F. W. 1986. Genetic drift and the loss of alleles versus heterozygosity. Zoo Biology 5:181-190.

Allendorf, F. W., G. Luikart, and S. N. Nelson. 2013. Conservation and the genetics of populations-2nd edition. Wiley-Blackwell, Oxford, United Kingdom.

Allendorf, F. W. and S. R. Phelps. 1981. Use of allele frequencies to describe population structure. Canadian Journal of Fisheries and Aquatic Sciences. 38:1507-1514.

Byorth, P. A. and J. P. Magee. 1999. As good grayling should. Montana Outdoors 30(6):7-11.

Cayer, E. and A. McCullough. 2013. Arctic grayling monitoring report 2012. Montana Fish, Wildlife & Parks.

Coleman, S. W. and A. G. Jones. 2011. Patterns of multiple paternity and maternity in fishes. Biological Journal of the Linnean Society 103:735-760.

Cornuet, J.-M. and G. Luikart. 1997. Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. Genetics 144:2001-2014.

Duftner, N., S. Koblmuller, S. Weiss, N. Medgyesy, and C. Sturnbauer. 2005. The impact of stocking on the genetic structure of European grayling (*Thymallus thymallus*) in two alpine rivers. Hydrobiologia 542:121-129.

Earthjustice. 2014. Conservation groups launch lawsuit to protect Montana grayling. http://earthjustice.org/news/press/2014/conservation-groups-launch-lawsuit- to-protect-Montana-grayling.

Evanno, G., S. Regnaut, and J. Goudet. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Molecular Ecology 14:2611-2620.

Everett, R. J. 1986. The population genetics of Arctic grayling (*Thymallus arcticus*) of Montana. Masters Thesis, University of Montana, Missoula, Montana.

Garant, G., J. J. Dodson, and L. Bernatchez. 2001. A genetic evaluation of mating system and determinants of individual reproductive success in Atlantic salmon (*Salmo salar* L). The Journal of Heredity 92:137-145.

Guo, S. W. and E. A. Thompson. 1992. Performing the exact test of Hardy-Weinberg proportions for multiple alleles. Biometrics 48:361-372.

Goudet, J., M. Raymond, T. deMeeus, and F. Rousset. 1996. Testing differentiation in diploid populations. Genetics 144:1933-1940.

Gum, B., R. Gross, and R. Kuehn. 2006. Discriminating the impact of recent human mediated stock transfer from historical gene flow on genetic structure of European grayling *Thymallus thymallus* L. Journal of Fish Biology 69(Supplement C):115-135.

Kalinowski, S. T. 2005. HP-RARE 1.0: a computer program for performing rarefaction on measures of allelic richness. Molecular Ecology Notes 5:187-189.

Kalinowski, S. T., A. P. Wagner, and M. L. Taper. 2006. ML-RELATE: a computer program for maximum likelihood estimation of relatedness and relationship. Molecular Ecology Notes 6:576-579.

Kaya, C. M. 1990. Status report on fluvial Arctic grayling (*Thymallus articus*) in Montana. Montana Fish, Wildlife & Parks.

Koskinen, M. T., J. Nilsson, N. J. Veselov, A. G. Potukin, E. Ranta, and C. R. Primmer. 2002. Microsatellite data resolve phylogeographic patterns in European grayling, *Thymallus thymallus,* Salmonidae. Heredity 88:391-401.

Krogstad, E. 2008. Early development of grayling in Lake Lesjaskoggvatn: a reciprocal transplant field experiment. Masters Thesis, University of Oslo, Oslo, Norway.

Leary, R. F. 1991. Establishment, maintenance, and use of a genetic reserve of Big Hole River Arctic grayling. University of Montana Wild Trout and Salmon Genetics Laboratory Report 91/5.

Leary, R. F. and F. W. Allendorf. 1993. California golden trout, founder effects, and lost genetic diversity. University of Montana Wild Trout and Salmon Genetics Laboratory Report 93/2.

Luikart, G. and J.-M. Cornuet. 1999. Estimating the effective number of breeders from heterozygote excess in progeny. Genetics 151:1211-1216.

Nakajima, M., N. Kanda, and Y. Fujio. 1991. Fluctuation of gene frequency in sub-populations originated from one guppy population. Nippon Suisan Gakkaishi-Bulletin of the Japanese Society of Scientific Fisheries 57:2223-2227.

Peakall, R. and P. E. Smouse (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Molecular Ecology Notes 6:288-295.

Peterson, D. P. and W. R. Ardren. 2009. Ancestry, population structure, and conservation genetics of Arctic grayling (*Thymallus articus*) in the upper Missouri River, USA. Canadian Journal of Fisheries and Aquatic Sciences 66:1758-1774.

Piry, S., A. Alapetite, J.-M. Cornuet, D. Paetkau, L. Baudouin, and A. Estoup. 2004. GENECLASS2: a software for genetic assignment and first-generation migrant detection. Journal of Heredity 95:536-539.

Piry, S., G. Luikart, and J.-M. Cornuet. 1999. BOTTLENECK: a computer program for detecting recent reductions in the effective population size using allele frequency data. The Journal of Heredity 90:502-503.

Pritchard, J. K., M. Stephens, and P. Donnelly. 2000. Inference of population structure using multilocus genotype data. Genetics 155:945-959.

Pritchard, J. K., W. Wen, and D. Faulsh. 2007. Documentation for *structure* software: version 2.2. http://pritch.bsd.uchicago.edu/software.

Pudovkin, A. I., O. L. Zhdanova, and D. Hedgecock. 1996. On the potential for estimating the effective number of breeders from heterozygote excess in progeny. Genetics 144:383-387.

Pudovkin, A. I., O. L. Zhdanova, and D. Hedgecock. 2010. Sampling properties of the heterozygote-excess estimator of the effective number of breeders. Conservation Genetics 11:759-771.

Redenbach, Z. and E. B. Taylor. 1999. Zoogeographical implications of variation in mitochondrial DNA of Arctic grayling (*Thymallus arcticus*). Molecular Ecology 8:23-35.

Rice, W. R. 1989. Analyzing tables of statistical tests. Evolution 43:223-225.

Rousset, F. 2008. GENEPOP'007: a complete re-implementation of the GENEPOP software for Windows and Linux. Molecular Ecology Resources 8:103-106.

Scott, W. B. and E. J. Crossman. 1973. Freshwater fishes of Canada. Bulletin 184, Fisheries Research Board of Canada, Ottawa, Canada.

Seamons, T. R., P. Bentzen, and T. P. Quinn. 2004. The mating system of steelhead, *Oncorhynchus mykiss*, inferred by molecular analysis of parents and progeny. Environmental Biology of Fishes 69:333-344.

Stamford, M. D., and E. B. Taylor. 2004. Phylogeographical lineages of Arctic grayling (*Thymallus arcticus*) in North America: divergence, origins and affinities with Eurasian *Thymallus*. Molecular Ecology 13:1533-1549.

Susnik, S., P. Berrebi, P. Dove, M. M. Hansen, and A. Snoj. 2004. Genetic introgression between wild and stocked salmonids and the prospects of using molecular markers in population rehabilitation: the case of the Adriatic grayling (*Thymallus thymallus* L. 1785). Heredity 93:273-292.

USFWS (United States Fish and Wildlife Service). 1994. Endangered and threatened wildlife and plants: finding on a petition to list the fluvial population of the Arctic grayling as endangered. Federal Register 59:37738-37741.

USFWS (United States Fish and Wildlife Service). 1996. Policy regarding the recognition of distinct vertebrate population segments under the Endangered Species Act. Federal Register 61:4722-4725.

USFWS (United States Fish and Wildlife Service). 2007. Endangered and threatened wildlife and plants: revised 12-month finding for upper Missouri River distinct population segment of fluvial Arctic grayling. Federal Register 72:20305-20314.

USFWS (United States Fish and Wildlife Service). 2010. Endangered and threatened wildlife and plants: revised 12-month finding for upper Missouri River distinct population segment of fluvial Arctic grayling. Federal Register 75:54708-54753.

USFWS (United States Fish and Wildlife Service). 2013. Endangered and threatened wildlife and plants: initiation of status review of Arctic grayling in the upper Missouri River system. Federal Register 78:70525-70527.

USFWS (United States Fish and Wildlife Service). 2014. Endangered and threatened wildlife and plants: revised 12-month finding on a petition to list the upper Missouri River distinct population segment of Arctic grayling as an endangered or threatened species. Federal Register 79:49384-49422.

Waples, R. S. and C. Do. 2008. Ldne: a program for estimating effective population size from data on linkage disequilibrium. Molecular Ecology Resources 8:753-756.

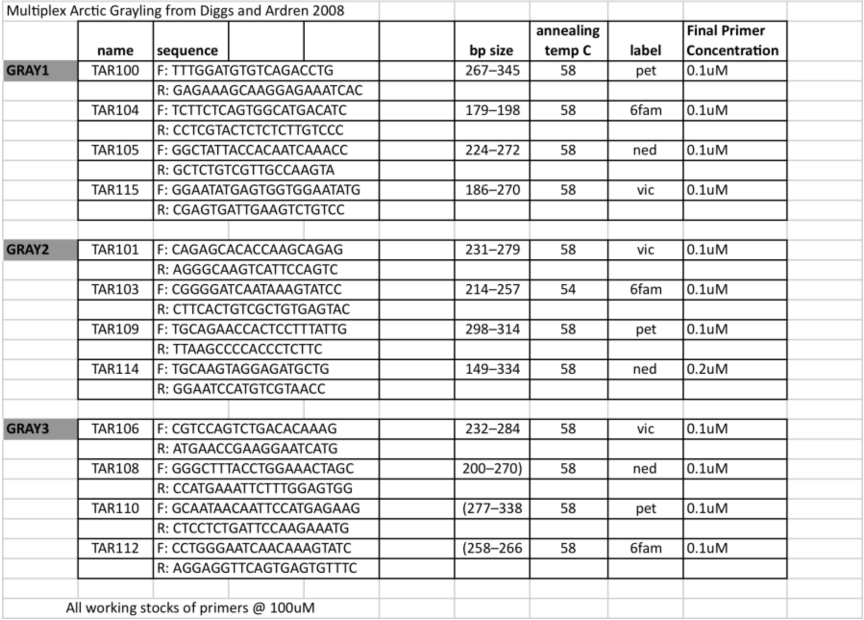
Weir, B. S. 1996. Genetic data analysis II. Sinauer Associates, Sunderland.

Weir, B. S., and C. C. Cockerham. 1984. Estimating F-statistics for the analysis of population structure. Evolution 28:1358-1370.

Table 1. Location, sample size (N), whether the population is believed to be native or introduced (status; ?=questionable), average expected heterozygosity (He), and allelic richness (AR) in samples of Arctic grayling from the upper Missouri River drainage, Montana. The total values for the Big Hole River were calculated using ten randomly chosen individuals per year class. Ruby River total values were calculated using all the individuals from each year class sample. Red Rock Creek total values were calculated using 20 randomly chosen individuals from each sample.

	N	Status	H_e	A_R
Big Hole (total)	70	Native	0.883	11.440
2007	50		0.878	12.440
2008	32		0.887	12.180
2009	131		0.882	11.950
2010	47		0.878	12.220
2011	66		0.885	12.230
2012	56		0.890	12.210
2013	50		0.887	12.260
Mussigbrod	50	Native	0.752	7.860
Miner	37	Native	0.800	5.620
Ruby (total)	81	Introduced	0.845	9.590
2010	43		0.814	9.260
2011	18		0.831	10.170
2012	20		0.843	10.800
Red Rock (total)	40	Native	0.766	9.170
2007	228		0.762	9.56
2008	100		0.751	9.27
Long	17	Native	0.667	7.160
O'Dell Creek	24	Native	0.731	7.740
Madison	23	Native	0.756	7.190
Bobcat	54	Introduced?	0.796	7.400
O'Dell Lake	50	Introduced	0.808	9.310
Deer	25	Introduced	0.708	6.120
Emerald	25	Introduced	0.805	8.340
Grayling	24	Introduced	0.697	5.260
Hyalite	25	Native?	0.676	5.800
Pintler	52	Native?	0.657	4.820

Table 2. Primers and protocol for microsatellites analyzed in Arctic grayling from the upper Missouri River drainage.



Multiplex Arctic Grayling from Diggs and Ardren 2008

	name	sequence	bp size	annealing temp C	label	Final Primer Concentration
GRAY1	TAR100	F: TTTGGATGTGTCAGACCTG	267–345	58	pet	0.1uM
		R: GAGAAAGCAAGGAGAAATCAC				
	TAR104	F: TCTTCTCAGTGGCATGACATC	179–198	58	6fam	0.1uM
		R: CCTCGTACTCTCTCTTGTCCC				
	TAR105	F: GGCTATTACCACAATCAAACC	224–272	58	ned	0.1uM
		R: GCTCTGTCGTTGCCAAGTA				
	TAR115	F: GGAATATGAGTGGTGGAATATG	186–270	58	vic	0.1uM
		R: CGAGTGATTGAAGTCTGTCC				
GRAY2	TAR101	F: CAGAGCACACCAAGCAGAG	231–279	58	vic	0.1uM
		R: AGGGCAAGTCATTCCAGTC				
	TAR103	F: CGGGGATCAATAAAGTATCC	214–257	54	6fam	0.1uM
		R: CTTCACTGTCGCTGTGAGTAC				
	TAR109	F: TGCAGAACCACTCCTTTATTG	298–314	58	pet	0.1uM
		R: TTAAGCCCCACCCTCTTC				
	TAR114	F: TGCAAGTAGGAGATGCTG	149–334	58	ned	0.2uM
		R: GGAATCCATGTCGTAACC				
GRAY3	TAR106	F: CGTCCAGTCTGACACAAAG	232–284	58	vic	0.1uM
		R: ATGAACCGAAGGAATCATG				
	TAR108	F: GGGCTTTACCTGGAAACTAGC	200–270)	58	ned	0.1uM
		R: CCATGAAATTCTTTGGAGTGG				
	TAR110	F: GCAATAACAATTCCATGAGAAG	(277–338	58	pet	0.1uM
		R: CTCCTCTGATTCCAAGAAATG				
	TAR112	F: CCTGGGAATCAACAAAGTATC	(258–266	58	6fam	0.1uM
		R: AGGAGGTTCAGTGAGTGTTTC				

All working stocks of primers @ 100uM

Table 3. Results of tests for gametic disequilibrium between pairs of loci in samples of Arctic grayling collected from the upper Missouri River drainage. Cells in red indicate statistically significant comparisons.

	Sample																						
Locus Pair	Big Hole 07	Big Hole 08	Big Hole 09	Big Hole 10	Big Hole 11	Big Hole 12	Big Hole 13	Ruby 10	Ruby 11	Ruby 12	Big Hole	Mussigbrod	Miner	Red Rock	Madison	Hyalite	Pintler	Ruby	Bobcat	Grayling	O'Dell	Deer	Emerald
1 100-104	🟥							🟥								🟥		🟥					
2 100-115										🟥													
3 104-115					🟥					🟥				🟥				🟥					
4 100-105				🟥	🟥			🟥		🟥												🟥	
5 104-105			🟥					🟥	🟥					🟥									
6 115-105										🟥													
8 104-101			🟥					🟥										🟥				🟥	
9 115-101	🟥									🟥													
10 105-101								🟥															
11 100-103																							
12 104-103			🟥		🟥																		
13 115-103			🟥							🟥								🟥					
14 105-103				🟥					🟥									🟥					
15 101-103					🟥													🟥					
16 100-109					🟥				🟥	🟥								🟥					
17 104-109			🟥															🟥					
18 115-109								🟥						🟥			🟥	🟥					
19 105-109			🟥						🟥	🟥							🟥	🟥					
20 101-109	🟥																						
21 103-109	🟥																						
22 100-114										🟥								🟥					
23 104-114					🟥			🟥										🟥					
24 113-114								🟥	🟥	🟥						🟥		🟥					
25 105-104					🟥			🟥		🟥													
26 101-114														🟥				🟥					
27 103-114	🟥		🟥		🟥			🟥	🟥	🟥								🟥					
28 109-114	🟥		🟥						🟥					🟥				🟥					
29 100-106			🟥					🟥		🟥								🟥					
30 104-106	🟥		🟥		🟥			🟥		🟥				🟥				🟥					
31 115-106								🟥		🟥				🟥		🟥		🟥					
32 105-106								🟥		🟥							🟥						
33 101-106								🟥		🟥				🟥				🟥					
34 103-106					🟥			🟥	🟥	🟥								🟥					
35 109-106														🟥		🟥		🟥					
36 114-106																		🟥				🟥	
37 100-108								🟥		🟥							🟥	🟥					
38 104-108					🟥			🟥															
39 115-109										🟥				🟥				🟥					
40 105-108								🟥															
41 101-108								🟥															
42 103-108			🟥		🟥					🟥								🟥					
43 109-108										🟥							🟥	🟥				🟥	
44 114-108								🟥		🟥								🟥					
45 106-108			🟥					🟥		🟥				🟥			🟥	🟥					
46 100-110								🟥	🟥									🟥					
47 104-110			🟥																				
48 115-110										🟥				🟥									
49 105-110										🟥													
50 101-110								🟥		🟥								🟥					
51 103-110			🟥																				
52 109-110														🟥				🟥					
53 114-110										🟥													
54 106-110			🟥					🟥		🟥				🟥									
55 108-110								🟥		🟥				🟥			🟥						
56 100-112					🟥			🟥		🟥								🟥					
57 104-112					🟥																		
58 115-112																		🟥					
59 105-112				🟥						🟥													
60 101-112														🟥									
62 109-112								🟥															
63 114-112			🟥															🟥					
64 106-112	🟥		🟥						🟥									🟥					
65 108-112	🟥			🟥				🟥															
66 110-112								🟥															

Table 4. Results of G test for significant allele frequency differences among samples of Arctic grayling collected from Red Rock Creek in 2007 and 2008, O’Dell Creek, and Long Creek.

```
-----------------------------------------------------
Locus          P-Value
-------------  --------
Tar100         0.13531
Tar104         0.18921
Tar115         0.09541
Tar105         0.02816
Tar101         0.60983
Tar103         0.6207
Tar109         0
Tar114         0.10177
Tar106         0.29592
Tar108         0.02843
Tar110         0.00267
Tar112         0.73106
```

Table 5. Estimates of F_{ST} between the combined 2007 and 2008 Red Rock Creek, O'Dell Creek, and Long Creek samples of Arctic grayling.

	Sample and F_{ST}		
	Red Rock	O'Dell	O'Dell
O'Dell	0.0067		
Long	0.0271		0.0177
	All	0.0152	

Table 6. Estimates of F_{ST} between samples of Arctic grayling collected from the upper Missouri River drainage, Montana.

Sample and F_{ST}

	Big Hole	Ruby	Red Rocks	Long	O'Dell Creek	Madison	Deer	Grayling	O'Dell Lake	Emerald	Mussigbrod	Miner	Bobcat	Hyalite
Ruby	0.0302													
Red Rocks	0.0795	0.1139												
Long	0.1117	0.1024	0.0226											
O'Dell Creek	0.0963	0.1472	0.0053	0.0177										
Madison	0.0724	0.1322	0.1836	0.2229	0.2027									
Deer	0.1036	0.0922	0.1200	0.1574	0.1329	0.1278								
Grayling	0.1080	0.0956	0.0938	0.1347	0.1146	0.1767	0.1350							
O'Dell Lake	0.0553	0.1472	0.0195	0.0589	0.0354	0.1257	0.0926	0.0861						
Emerald	0.0527	0.0883	0.0721	0.1175	0.0911	0.0996	0.1023	0.1088	0.0355					
Mussigbrod	0.0967	0.1479	0.1997	0.2354	0.2154	0.1610	0.2137	0.2231	0.1656	0.1506				
Miner	0.0814	0.1472	0.1777	0.2170	0.1970	0.1551	0.1990	0.2078	0.1517	0.1423	0.1080			
Bobcat	0.0882	0.1240	0.0693	0.1080	0.0774	0.1176	0.1182	0.1217	0.0427	0.0456	0.1638	0.1498		
Hyalite	0.1042	0.1660	0.0959	0.1250	0.1166	0.1984	0.1721	0.1577	0.0913	0.1120	0.2373	0.2160	0.1302	
Pintler	0.1272	0.0945	0.2075	0.2496	0.2270	0.1789	0.1911	0.2356	0.1725	0.1120	0.1888	0.2076	0.1851	0.2390

Table 7. Results of G test for significant allele frequency differences among samples of the 2007-2013 year classes of Arctic grayling collected from the Big Hole River.

```
-----------------------------------------------------
Locus          P-Value
-------------  --------
Tar100         0.03767
Tar104         0.00573
Tar115         0.02017
Tar105         0.74919
Tar101         0.04579
Tar103         0.23528
Tar109         0.02381
Tar114         0
Tar106         0.02379
Tar108         2e-05
Tar110         0
Tar112         0.07866
```

Table 8. Estimates of F_{ST} between samples of the 2007-2013 year classes of Arctic grayling collected from the Big Hole River.

	Big Hole Year Class					
	2007	2008	2009	2010	2011	2012
2008	0.0050					
2009	0.0060	-0.0014				
2010	0.0059	0.0008	0.0023			
2011	0.0036	0.0020	0.0039	0.0030		
2012	0.0021	0.0014	0.0015	0.0023	0.0015	
2013	0.0025	0.0027	0.0026	0.0028	0.0048	-0.0007
	All	0.0027				

Table 9

95% confidence intervals and point estimates of effective number of breeders for the 2007-2013 year class samples of Arctic grayling from the Big Hole River.

Lowest Allele Frequency Used	0.05	0.02	0.01
Year Class	95% Confidence Interval and point estimate in parentheses		
2007	61.6-106.2 (78.8)	97.4-155.0 (120.2)	124.6-206.3 (156.2)
2008	72.3-380.3 (125.6)	82.1-204.6 (118.7)	156.2-1146.9 (280.2)
2009	58.0-74.7 (65.7)	81.4-97.6 (88.9)	92.6-109.3 (100.4)
2010	83.6-192.4 (118.4)	90.1-145.1 (111.8)	125.4-228.8 (163.0)
2011	68.8-115.6 (63.9)	79.4-108.6 (63.9)	85.9-115.6 (64.0)
2012	144.8-886.2 (265.5)	205.9-740.3 (326.9)	219.7-624.0 (328.3)
2013	163.1-2240.6 (314.2)	283.4-6705.2 (553.3)	373.3-Infinity (884.8)

Table 10

Number of male and female Arctic grayling from the Big Hole River spawned to establish a captive broodstock.

Year Spawned	Number of Fish Spawned	
	Males	Females
1988	9	4
1990	18	11
1991	14	7
1992	18	8
2010	19	6
2011	2	1
2012	8	8
2013	12	12
Total	100	57

Table 11. Results of G test for significant allele frequency differences among samples of the 2010-2012 year classes of Arctic grayling collected from the Ruby River.

```
-----------------------------------------------------
Locus          P-Value
-------------  --------
Tar100         0.27415
Tar104         0.03658
Tar115         0
Tar105         0
Tar101         0.00537
Tar103         0.00652
Tar109         0.00521
Tar114         0.02197
Tar106         0.06384
Tar108         0.13783
Tar110         0.01399
Tar112         0.16258
```

Table 12. Estimates of F_{ST} between three year class samples of Arctic graying collected from the Ruby River.

	Ruby Year Class	
	2010	2011
2011	0.0198	
2012	0.0231	0.0041
	All	0.0183

Table 13. Results of G test for significant allele frequency differences between samples of Arctic grayling collected from the Big Hole and Ruby River.

```
--------------------------------------------------------
Locus           P-Value
-------------   --------
Tar100          0
Tar104          0
Tar115          0
Tar105          0
Tar101          0
Tar103          0
Tar109          0
Tar114          0
Tar106          0
Tar108          0
Tar110          0
Tar112          0
```

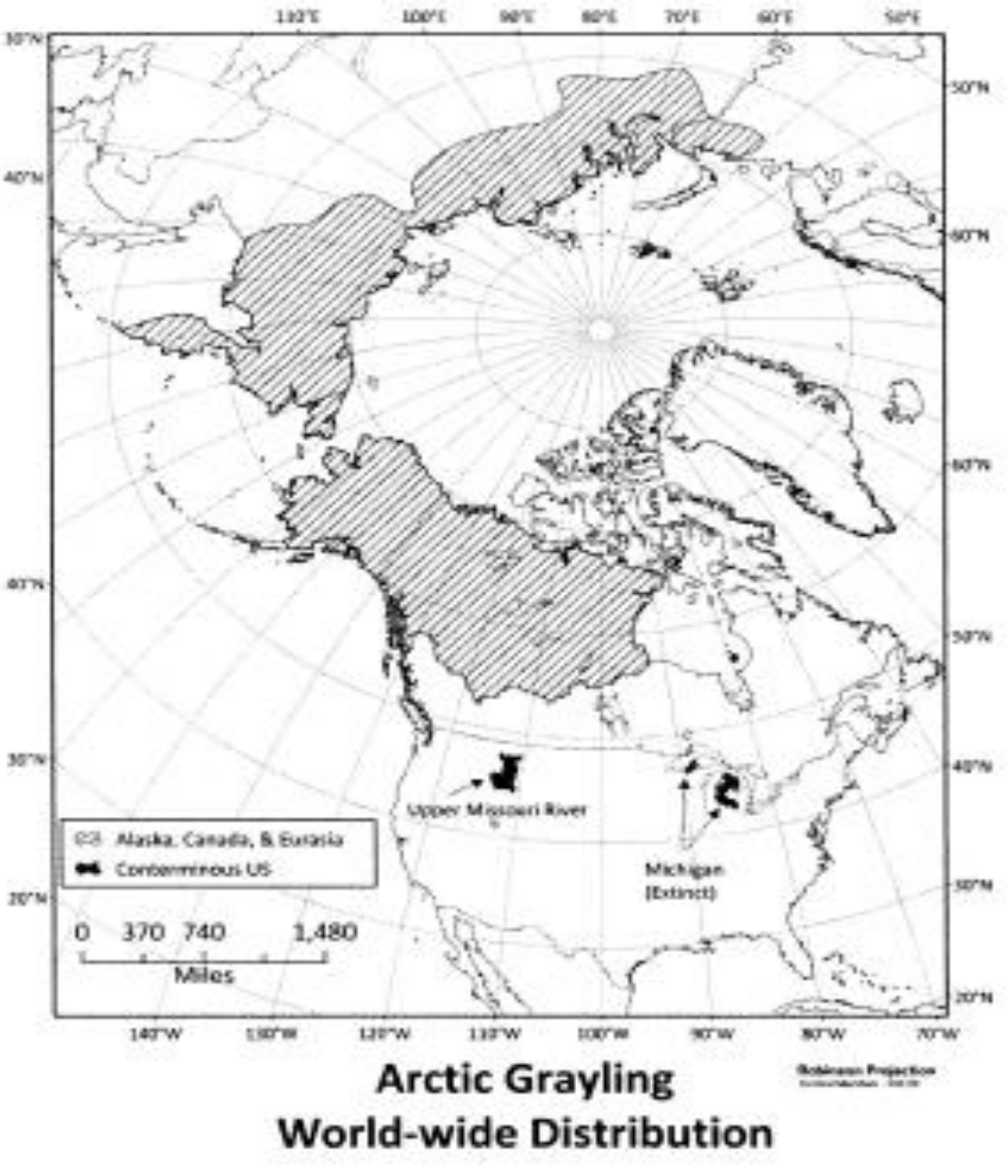


Figure 1. Historic distribution of Arctic grayling. Note the occurrence of disjunct populations in Michigan and the upper Missouri river drainage in Montana and northwestern Wyoming.

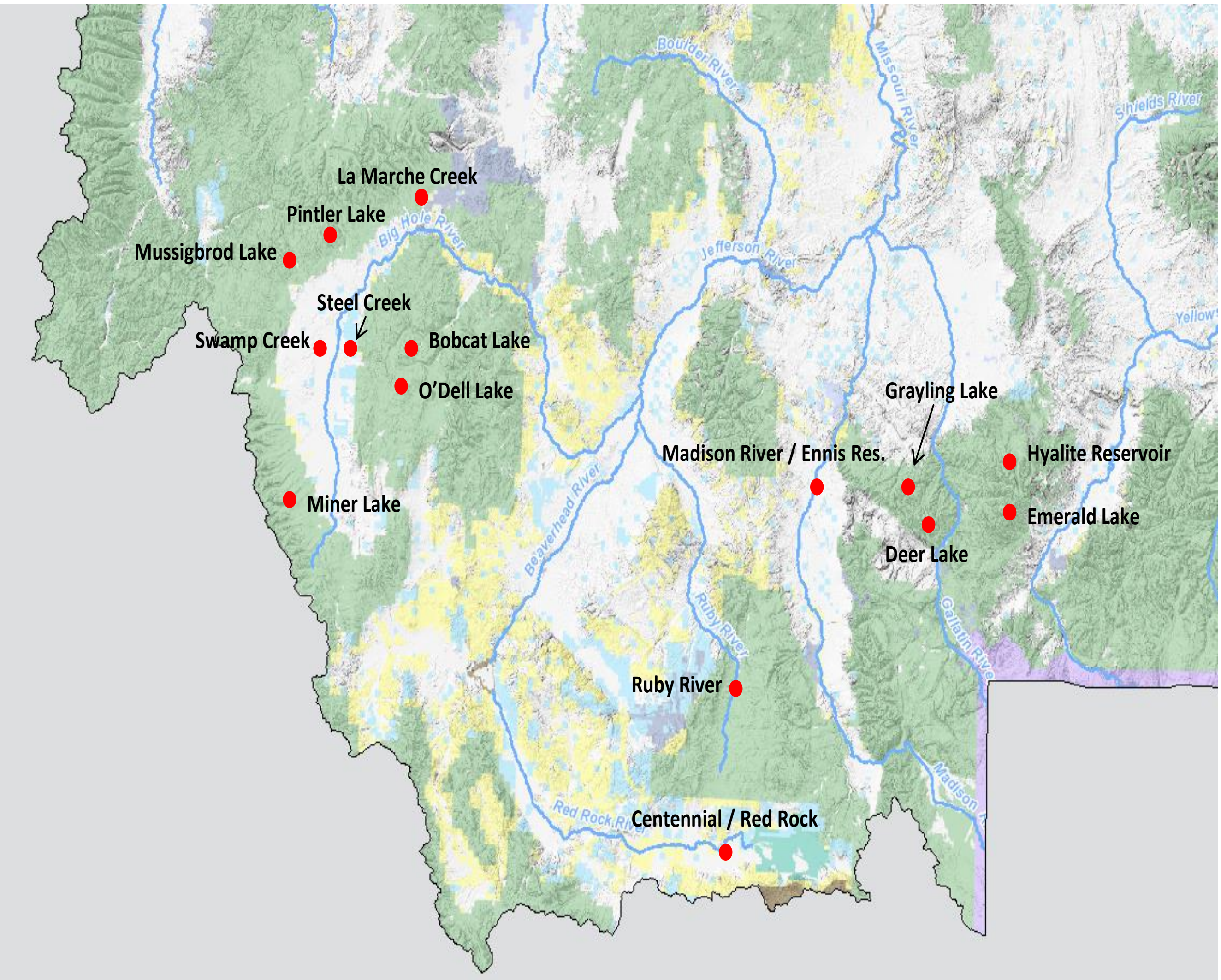


Figure 2. Approximate location of Arctic grayling samples used in this study. Centennial/Red Rock represents the approximate location of the Long Creek, Red Rock Creek, and O'Dell Creek samples. Swamp Creek, Steel Creek, and LaMarche Creek represent approximate location of sample sites in the Big Hole River drainage.

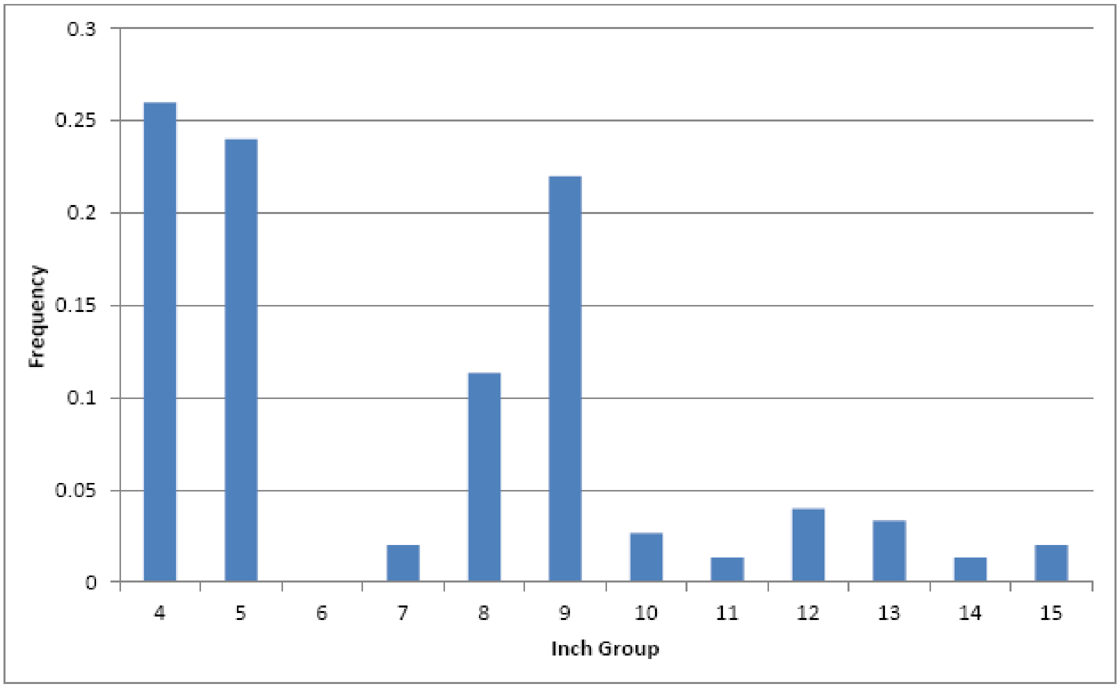


Figure 3. Length frequency histogram of Arctic grayling collected from the Big Hole River during the fall of 2011. The four and five inch fish represent young of the year individuals.

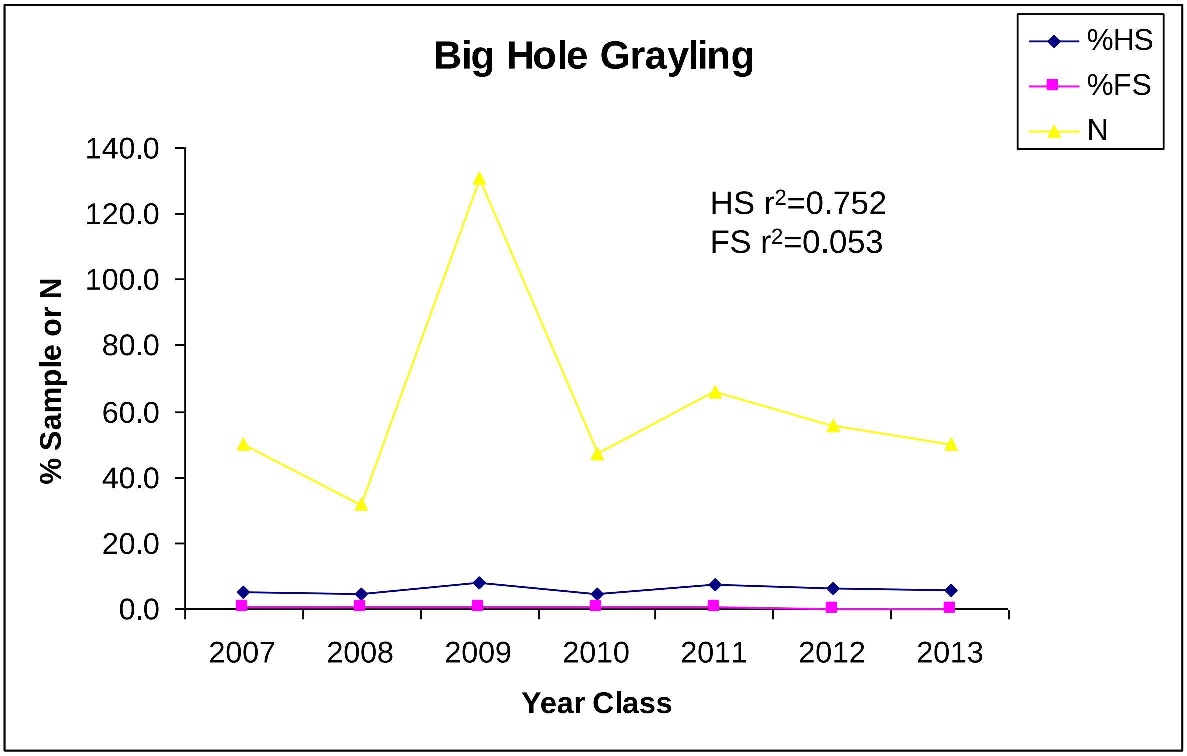


Figure 4. Regression between percent of sample estimated to be half (HS) or full siblings (FS) and sample size (N) in the 2007-2013 year classes of Arctic grayling collected from the Big Hole River.

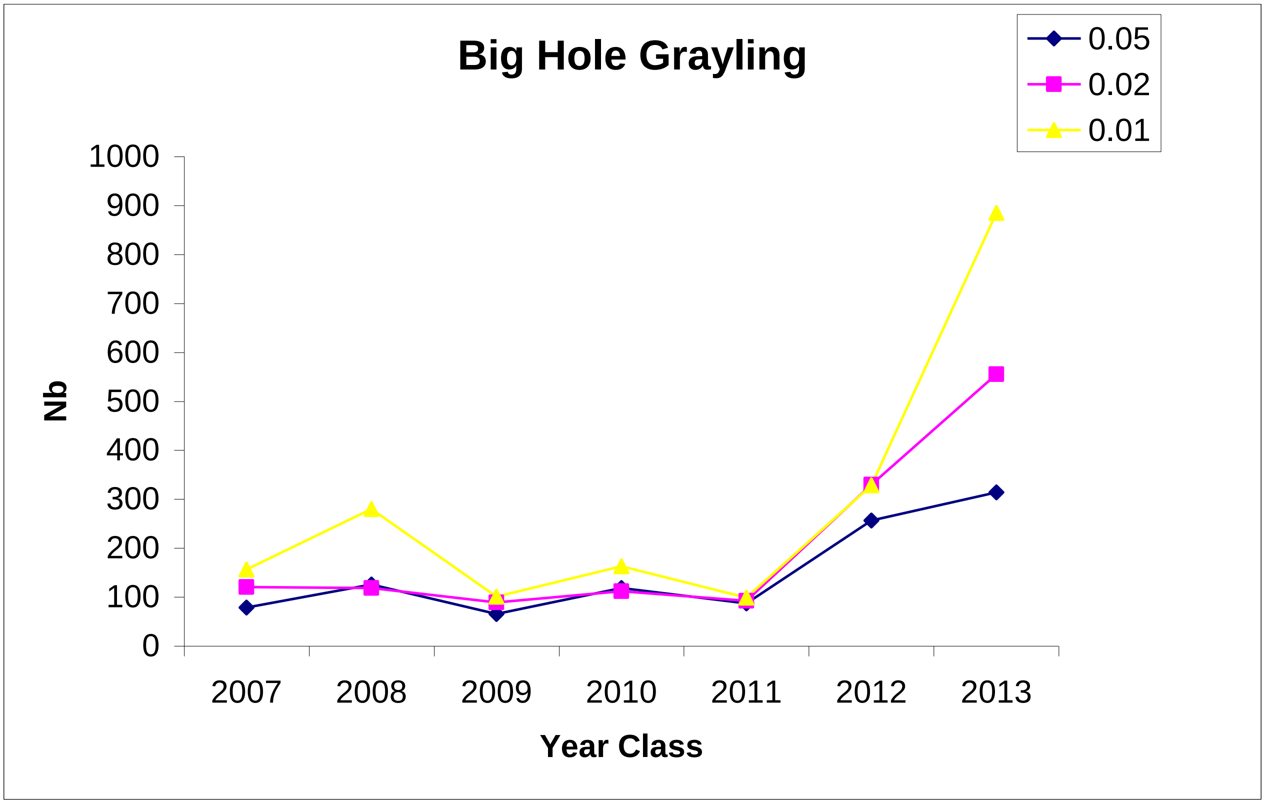


Figure 5. Estimates of the effective number of breeders (N_b) in the 2007-2013 year classes of Arctic grayling collected from the Big Hole River. Analyses were conducted using all alleles at a frequency greater than 0.01, 0.02, or 0.05. Note as the number of low frequency alleles in the data increases (lower cut off level) the N_b tends to increase.

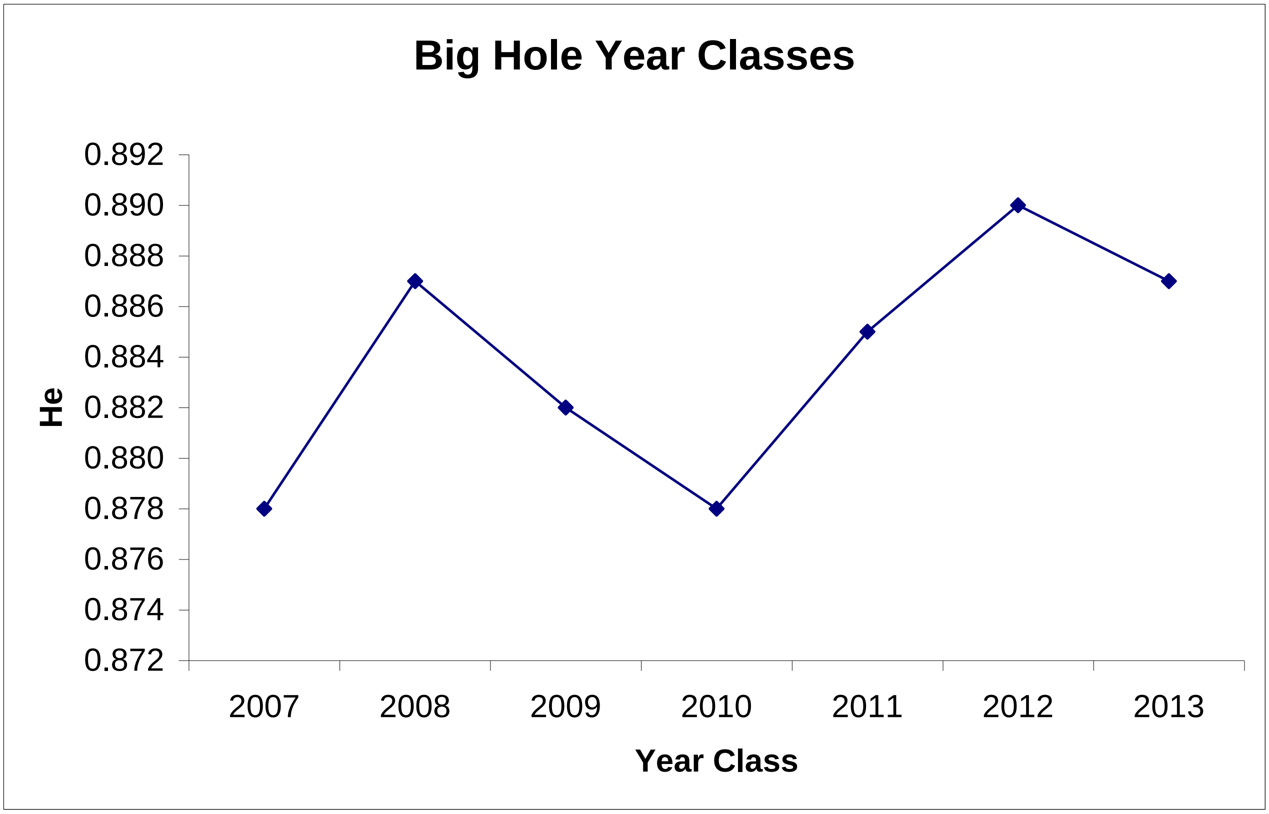


Figure 6. Average expected heterozygosity (H_e) among samples of the 2007-2013 year classes of Arctic grayling collected from the Big Hole River.

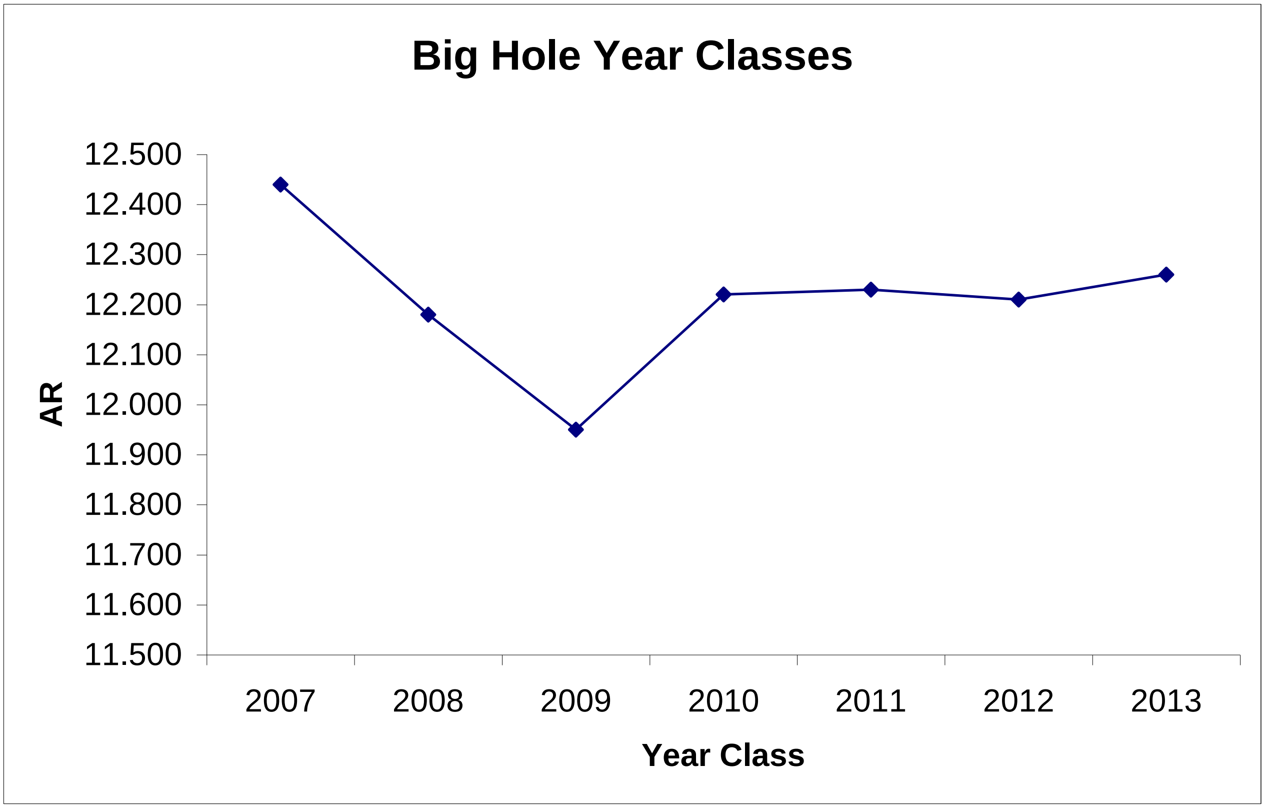


Figure 7. Allelic richness (A_R) among samples of the 2007-2013 year classes of Arctic grayling collected from the Big Hole River.

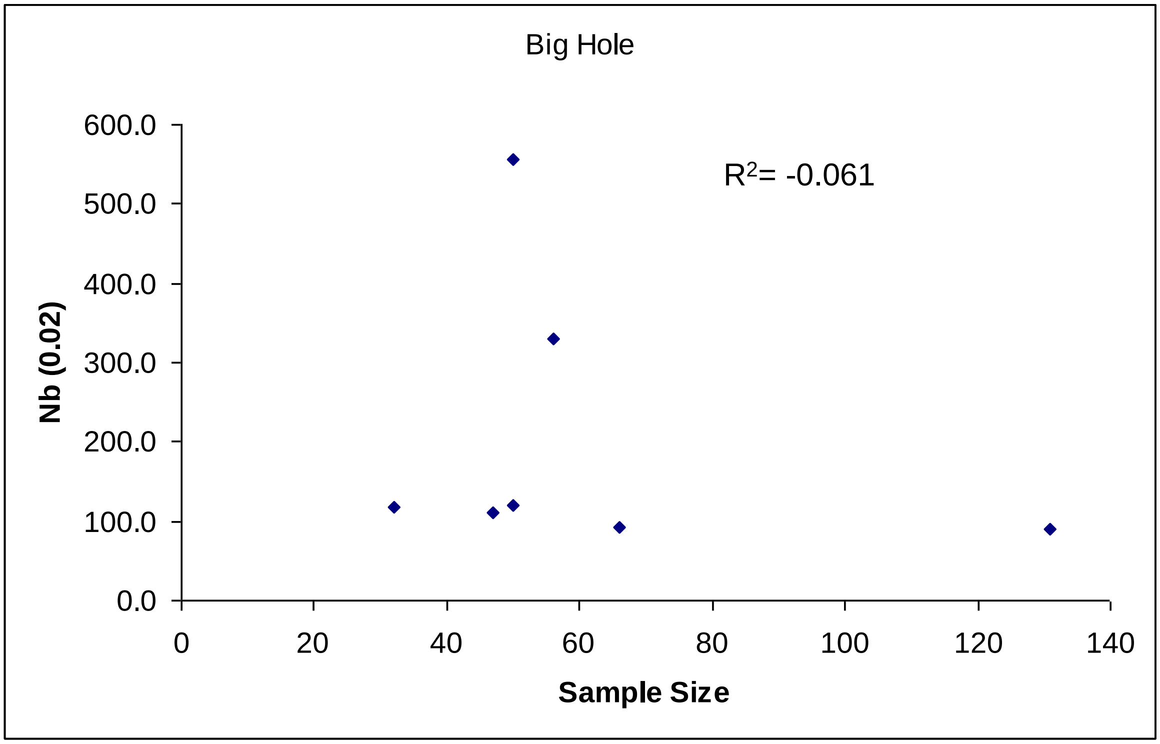


Figure 8. Regression of effective number of breeders (Nb) on sample size for samples of the 2007-2013 year classes of Arctic grayling collected from the Big Hole River. N_b was estimated using all the alleles at a frequency greater than 0.02.

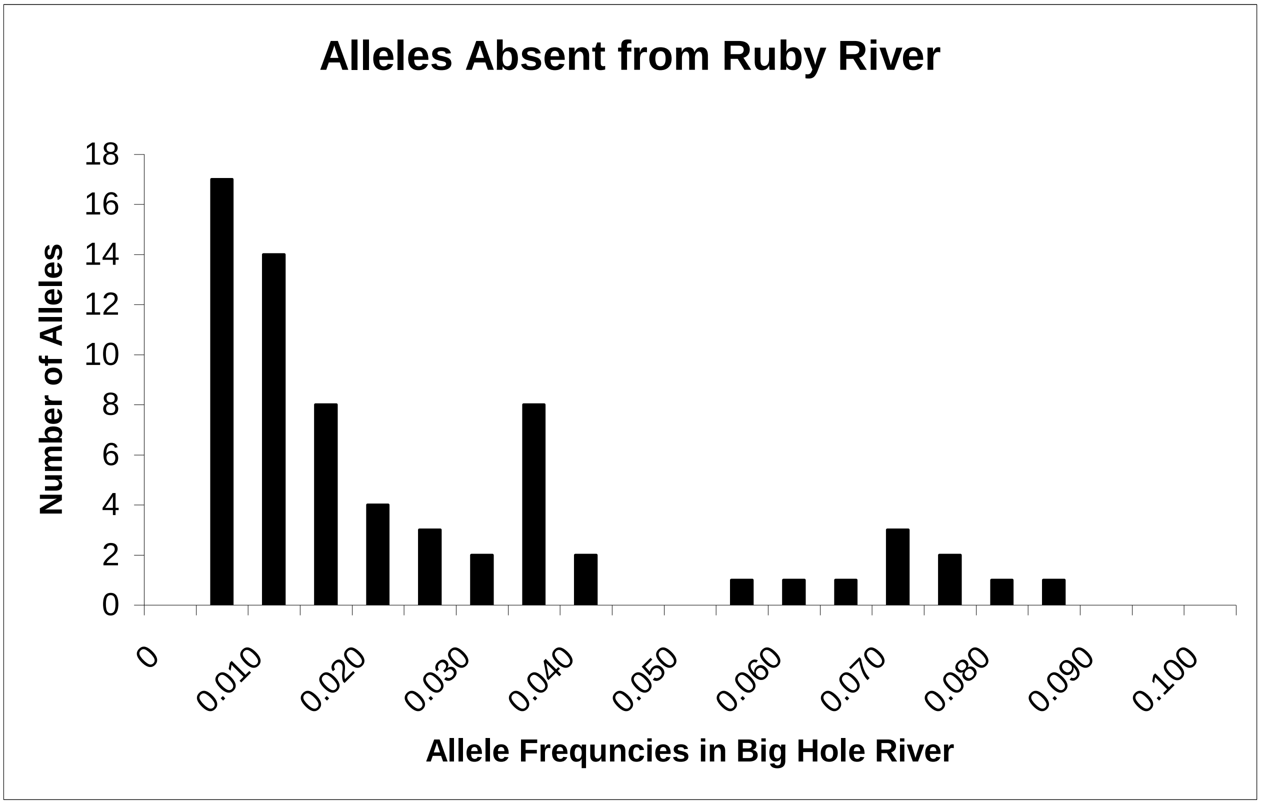


Figure 9. Frequency of alleles detected in the Big Hole River sample but, not detected in the Ruby River sample Note the Ruby River population was established using gametes collected from the Big Hole River broodstock.

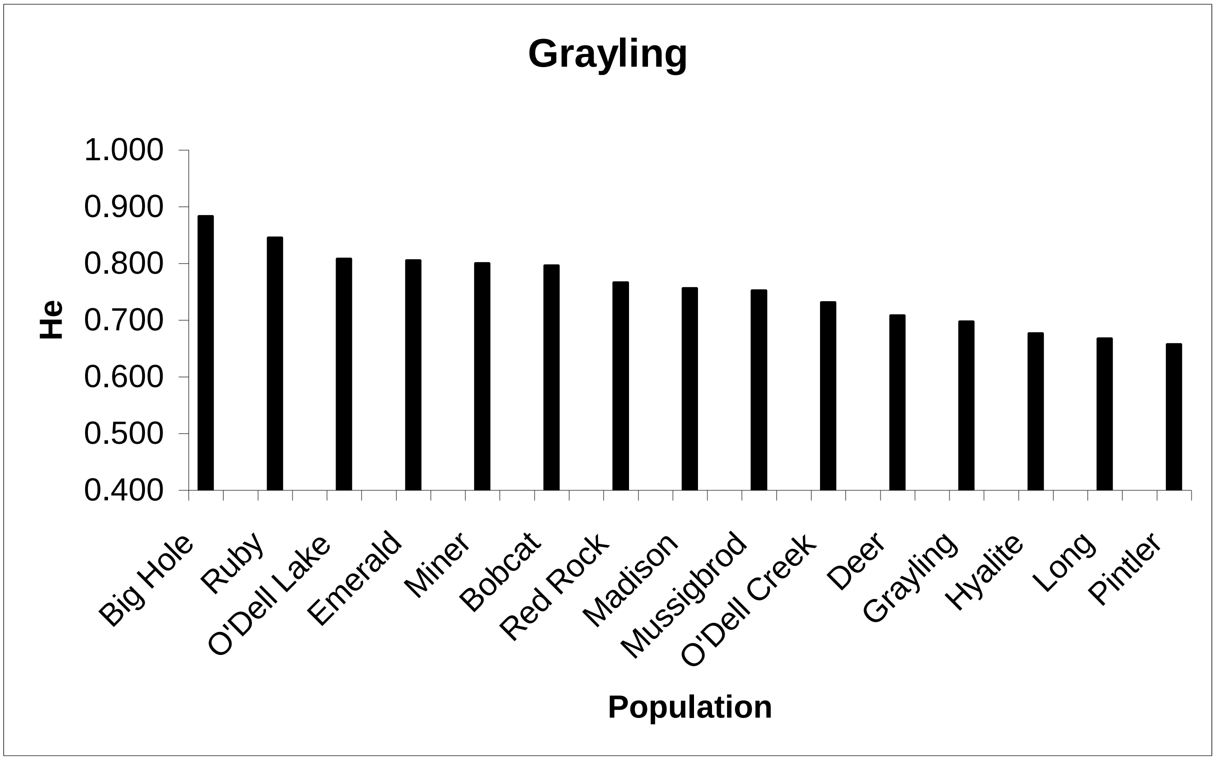


Figure 10. Estimates of average expected heterozygosity (H_e) in samples of Arctic grayling collected from the upper Missouri River drainage.

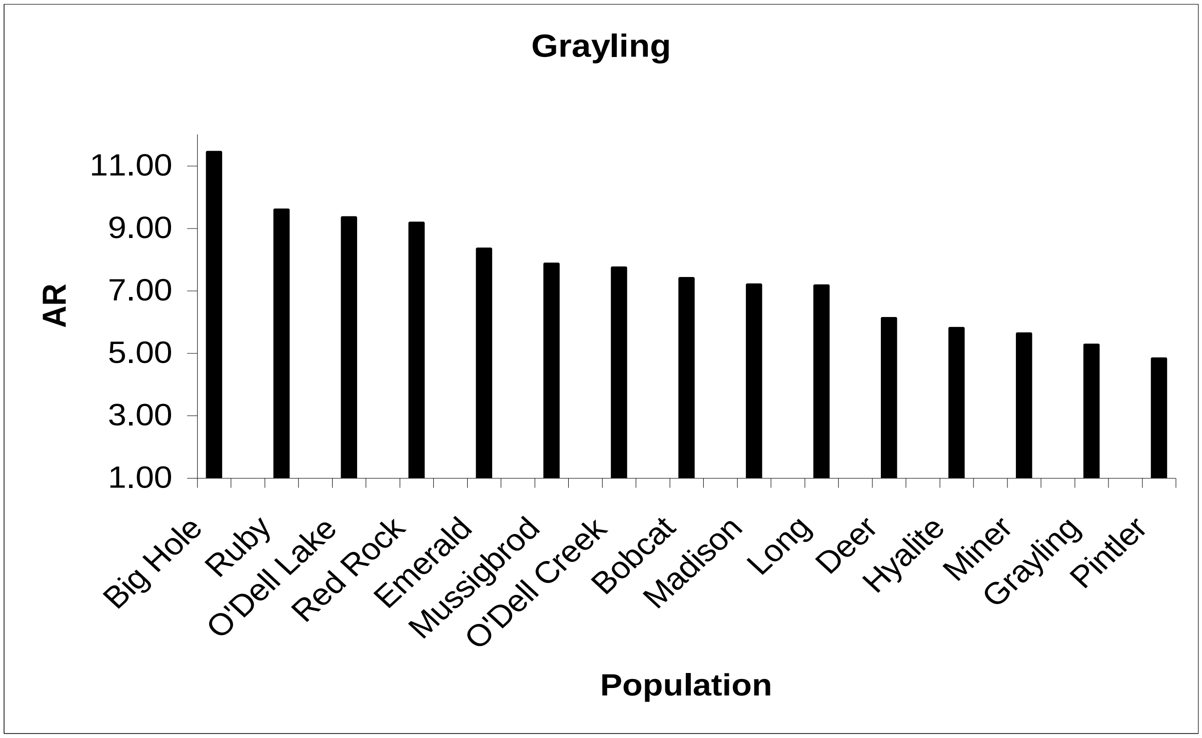


Figure 11. Estimates of allelic richness (A_R) in samples of Arctic grayling collected from the upper Missouri River drainage.

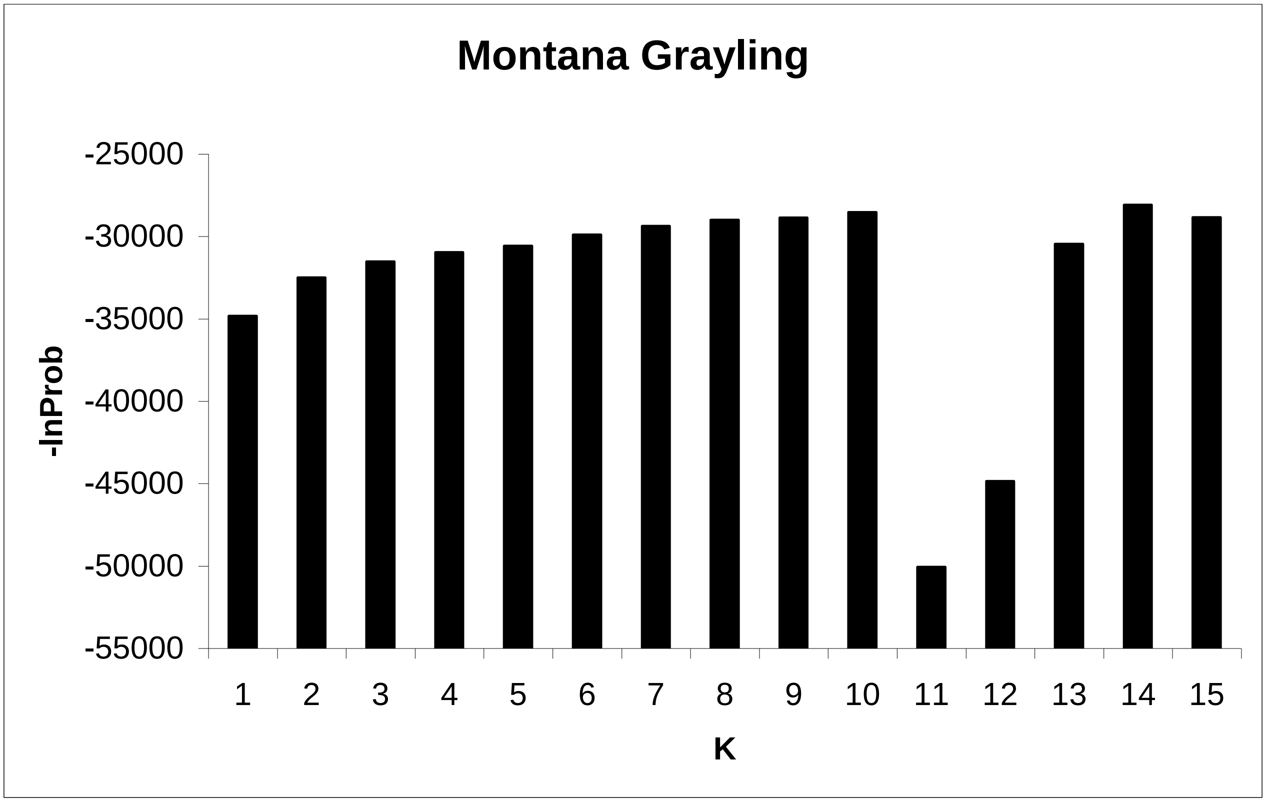


Figure 12. Mean probability values of five iterations of STRUCTRE with K=1-15 using 15 samples of Arctic grayling collected from the upper Missouri River drainage, Montana.

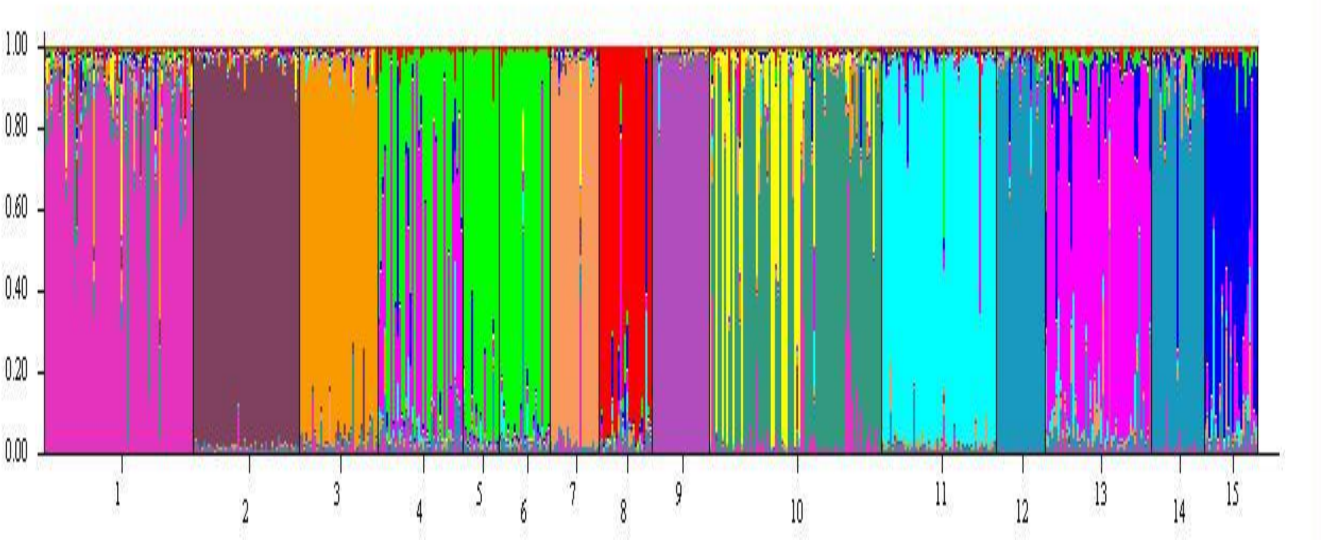


Figure 13. Results of STRUCTURE with K=13. Samples: Native 1=Big Hole River, 2=Mussigbrod Lake, 3=Miner Lake, 4=Red Rock Creek, 5=Long Creek, 6=O'Dell Creek, 7=Madison River, 8=Hyalite Reservoir, 9=Pintler Lake; Introduced 10=Ruby River, 11=Bobcat Lake, 12=Grayling Lake, 13=O'Dell Lake, 14=Deer Lake, 15=Emerald Lake.

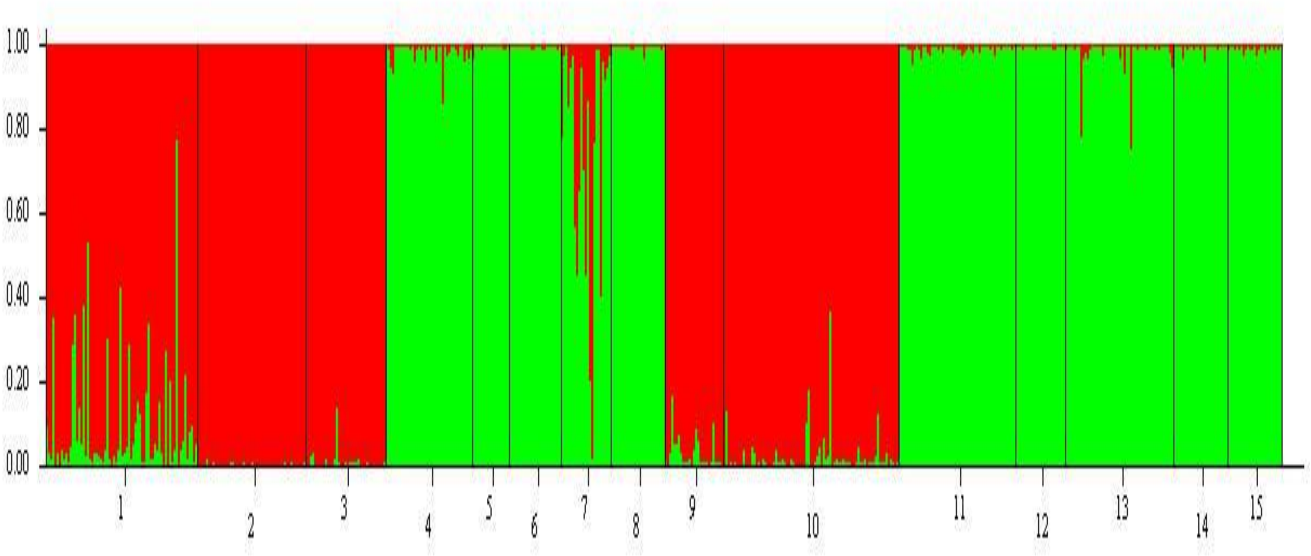


Figure 14. Results of STRUCTURE with K=2. Samples: Native 1=Big Hole River, 2=Mussigbrod Lake, 3=Miner Lake, 4=Red Rock Creek, 5=Long Creek, 6=O'Dell Creek, 7=Madison River, 8=Hyalite Reservoir, 9=Pintler Lake; Introduced 10=Ruby River, 11=Bobcat Lake, 12=Grayling Lake, 13=O'Dell Lake, 14=Deer Lake, 15=Emerald Lake.

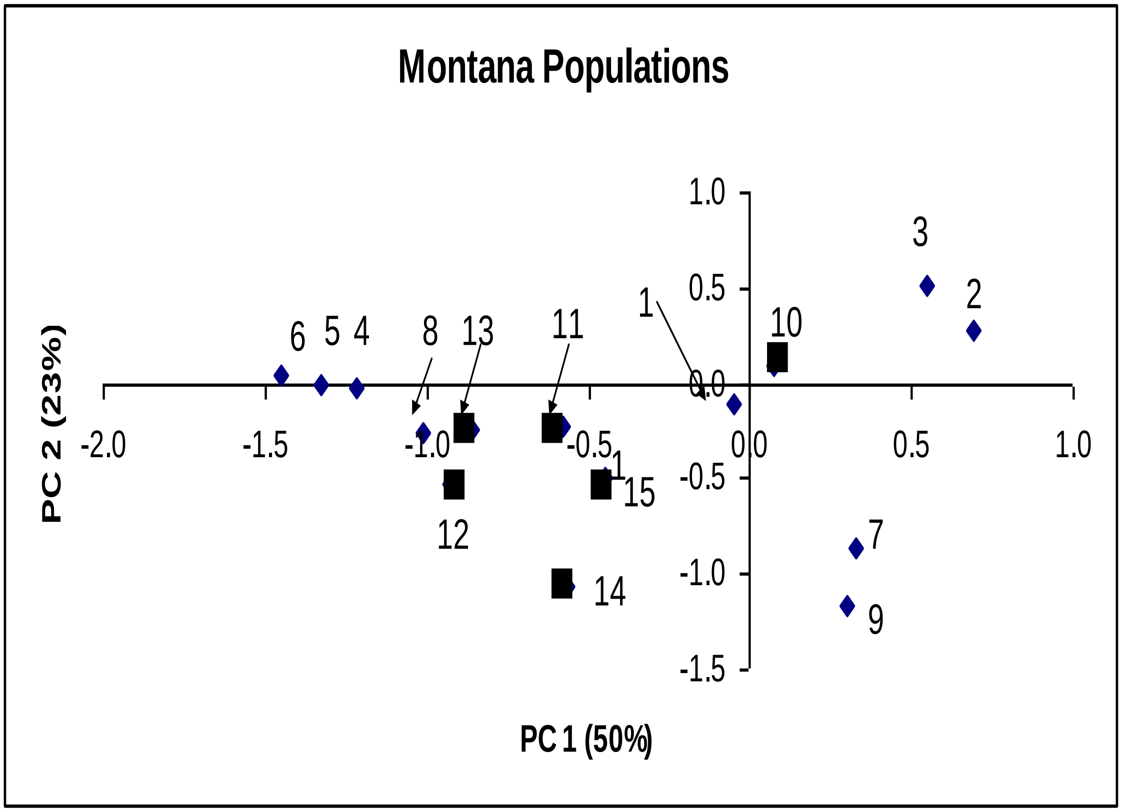


Figure 15. Location of samples along the first two principal components axes. Samples: Native (diamonds) 1=Big Hole River, 2=Mussigbrod Lake, 3=Miner Lake, 4=Red Rock Creek, 5=Long Creek, 6=O’Dell Creek, 7=Madison River, 8=Hyalite Reservoir, 9= Pintler Lake; Introduced (squares) 10=Ruby River, 11=Bobcat Lake, 12=Grayling Lake, 13=O’Dell Lake, 14=Deer Lake, 15=Emerald Lake.