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ARTICLE

Genetic monitoring of steelhead in the Klickitat River to estimate productivity, straying, and migration timing

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Abstract

Objective: Salmonids with a complex life history variation present challenges for conservation management, but genetic approaches alongside fisheries monitoring can address questions regarding the viability of the natural populations.

Methods: We genotyped adult ($n = 3108$) and juvenile ($n = 2624$) samples of steelhead *Oncorhynchus mykiss* that were collected in the Klickitat River, Washington, USA at traps in the lower drainage to examine tributary level productivity, straying from outside sources, and variation in adult migration timing.

Result: Genetic assignment of steelhead from this system indicated that the majority were produced within or near tributaries of the middle Klickitat River (juvenile mean = 72.8%; adult mean = 87.3%). Analyses with parentage-based tagging identified that most hatchery-origin adults were assigned to the Skamania Hatchery (80.8%), as expected, since this has been the release stock for decades within the Klickitat River drainage. Hatchery-origin adults were also identified from programs operating outside the Klickitat River, which were primarily strays from Snake River hatcheries. Most natural-origin steelhead were assigned to the Klickitat River, but there were also natural-origin fish identified as strays from other regions of the Columbia River (22.3% of natural returns). We also examined genes known to be associated with migration timing in adult steelhead observed at the trap and observed a strong relationship between migration date and alleles for early and late migration, but individual outliers were detected across seasons.

Conclusion: Our results indicate that genetic variation of steelhead in the Klickitat River has been influenced by hatchery programs as well as natural-origin straying from other subbasins, but genetic diversity remains high throughout the subbasin, and both early and late migration alleles are maintained. The genetic diversity present in Klickitat River steelhead may enable this Endangered Species Act listed (threatened) species to better adapt to stochastic environmental conditions compared to less diverse populations.

KEYWORDS

genetics, migration timing, population dynamics, steelhead

INTRODUCTION

Contemporary patterns of diversity in Pacific salmon have emerged as the result of the persistence of ancestral lineages, recolonization from glacial refugia, and further evolution throughout the Holocene (Waples et al. 2008). In recent centuries, anthropogenic effects have degraded ecosystems for Pacific salmon and played a key role in reducing diversity, resilience, and population sizes over time (e.g., National Research Council 1996; English et al. 2006; Moore et al. 2014). Populations of steelhead *Oncorhynchus mykiss* (anadromous Rainbow Trout) are an example of a crucial cultural, economic, and ecological resource, which is declining in many regions throughout its geographic range due to anthropogenic effects (Gustafson et al. 2007). Steelhead population sizes were decimated by overharvest and habitat degradation (logging, mining, agricultural practices) during the 1800s and the initiation of the hydrosystem furthered their declines by disruption of migratory routes (Poff et al. 2007). More recently, climate change (Crozier et al. 2008) is a major factor contributing to these declines, as well as decreases in fitness, potentially due to introgression between native populations and hatchery stocks (Chapman 1986; Meehan 1991). Long-term viability of steelhead entails multiple factors including maintaining phenotypic and genetic diversity of distinct populations (McElhany et al. 2000).

Genetic lineages of steelhead in the Columbia River basin have been previously designated as coastal and inland (e.g., Utter et al. 1980; Blankenship et al. 2011; Micheletti et al. 2018a). The two lineages are geographically separated by the Cascade Mountains and populations within the Klickitat River subbasin are genetically intermediate between the coastal and inland lineages (Quinn 2018; Collins et al. 2020). A long history of natural introgression and high diversity of life history traits has characterized populations of salmonids in the Cascade Crest region. Steelhead populations in this dynamic region of the Columbia River Gorge are included in the threatened Middle Columbia River evolutionarily significant unit (ESU) with protection under the Endangered Species Act (Busby et al. 1996). The Klickitat River is an important habitat for steelhead that maintains highly diverse populations across the drainage (Narum et al. 2008). Steelhead populations in the Klickitat River represent a mix of major lineages and populations exhibit further ecotypic diversity with bimodal migration timings (Hess et al. 2016b; Micheletti et al. 2018b). Adult steelhead in the Klickitat River spawn in the spring, but there are two modes of distinct migration timing into freshwater, with one ecotype returning as early as summer of the previous year before spawning (often known as the summer-run) and the other ecotype as late as winter/spring just before spawning (often known as the winter-run; Quinn et al. 2015; Hess et al. 2016b). Landscape features in the Klickitat River such as high gradient reaches and waterfalls can further separate populations of steelhead (Narum et al. 2006, 2008).

Impact statement

Steelhead are ecologically, culturally, and economically significant and there are steelhead populations listed as threatened under the Endangered Species Act. To assist conservation efforts, we evaluated the genetic diversity of these populations as a measure of their fitness and ability to adapt to environmental changes.

In addition to natural populations of steelhead in the Klickitat River, steelhead from the Skamania Hatchery have been stocked in the Klickitat River since 1961 in the drainage to allow for harvest opportunity, which would otherwise be very limited. There have also been hatchery strays (hatchery-origin adults from outside sources) detected in the Klickitat River (Narum et al. 2006) and it is uncertain what effect that either the Skamania Hatchery stocks from the Washougal River drainage or other outside sources of hatchery-origin adults have on the natural-origin population. These hatchery facilities in the Columbia River basin exist to mitigate the impacts on fish habitat from construction of dams and other human developments. Although this mitigation purpose of hatcheries is key to salmonid management goals, there are potentially undesirable effects. Previous studies suggest that steelhead may be particularly susceptible to reduced fitness when interbreeding with hatchery-origin fish compared to other salmonid species (reviewed in Koch and Narum 2021). Summer-run hatchery steelhead from the Skamania Hatchery can also influence the genetic structure of steelhead populations due to possible overlapping spawning timing with natural-origin summer steelhead, but limited gene flow has been previously detected (Narum et al. 2006). Skamania steelhead were originally derived from steelhead from the Washougal and Klickitat rivers and have been strongly selected for early spawning times, which increased temporal reproductive isolation due to earlier migration timing (Ayerst 1977; Crawford 1979; Chilcote et al. 1986; Kostow et al. 2003). Nonnative strains of resident Rainbow Trout have also been stocked in the Klickitat River from hatcheries (i.e., McCloud River, Goldendale, and Mt. Whitney strains) (Crawford 1979) but remain distinct from native populations in the subbasin (Narum et al. 2008). Hatchery straying from outside basins has not been fully described or quantified, which has precluded any ability to understand potential influences on traits in the natural population (Narum et al. 2006).

Adult migration timing is an important trait influencing fitness of steelhead throughout their geographic range (Carlson and Seamons 2008; Quinn et al. 2015), and both early summer-run and later winter-run ecotypes are present in the natural-origin steelhead population of the Klickitat River (Hess et al. 2016b). The summer-run Skamania source stock have the potential to influence both the natural-origin early and late migrating steelhead, given their overlapping spawning. Additionally, any hatchery strays from other sources in the Columbia River could be mixtures of migration ecotypes (Hess et al. 2016b) and would also have the potential to influence the composition of migration ecotypes in the natural-origin Klickitat River steelhead populations. An association between migration timing and a genomic region of major effect has been previously identified in steelhead (Hess et al. 2016b; Prince et al. 2017; Micheletti et al. 2018b; Collins et al. 2020; Willis et al. 2020). The terms "early" and "late" are used when describing migration phenotypes and their associated alleles when describing genotypes to differentiate migration timing phenotypes and associated genotypes (Waples et al. 2022). Markers have been developed for the *greb1L* and *rock1* gene region on chromosome 28 (Collins et al. 2020; Willis et al. 2020), which can classify steelhead into these migration ecotypes and contribute information for genetic monitoring of conservation units and fisheries management (e.g., Waples and Lindley 2018). The predictive ability (i.e., strength of the genotype-by-phenotype associations) of these genetic classifications of migration ecotypes can be determined by pairing them with the physical movements of steelhead as they migrate through the Columbia River (Willis et al. 2020) or return to spawn in subbasins such as the Klickitat River (Micheletti et al. 2018b). If introgression from hatchery-origin stocks had a quantifiable influence on the genetic composition of natural-origin steelhead of the Klickitat River, there would be potential for decreased fitness if this influence was associated with a shift in genetic and phenotypic traits related to migration timing. The presence of hatchery-origin adults can be determined because most are physically marked with an adipose fin clip, but the spawning contribution of these hatchery-origin adults to a natural population can have longer-lasting effects through introgression and these effects can only be identified via genetic monitoring.

In this study, we examined the diversity of steelhead in the Klickitat River by genotyping adults and juveniles that were collected at traps in the lower portion of the drainage to estimate population productivity, straying from outside sources, and variation in adult migration timing in the Klickitat River. We addressed the following primary questions: (1) Do particular areas within the Klickitat River subbasin produce more natural-origin steelhead than others? (2) Do hatchery-origin steelhead that are stocked, or stray from outside sources, into this system interbreed with natural populations and influence genetic diversity? (3) Has adult migration timing in natural-origin steelhead been influenced by introgression with summer-run hatchery fish? Overall, this study provides results from an intensive genetic monitoring program that provides information to assist with conservation and recovery of steelhead diversity in this system.

METHODS

Sample collection

Samples were collected from the Klickitat River (Figure 1) to address various objectives including: juvenile natural-origin steelhead from tributaries to create a local genetic baseline for stock assignment; a mixture of natural-origin juvenile steelhead out-migrating through the main-stem Klickitat River to estimate local productivity and introgression; and adult steelhead at the Lyle Falls trap to evaluate the mixture of stocks returning annually (both hatchery- and natural-origin). Samples were analyzed with a combination of genetic stock identification (GSI) and parentage-based tagging (PBT) methods to address objectives with the workflow illustrated in Figure 2.

For the genetic baseline, samples were collected from specific tributary locations throughout the subbasin between 2011 and 2018 to construct a local baseline to assign unknown fish with GSI methods within the Klickitat River. These local baseline samples were collected with electrofishing methods from tributaries throughout the subbasin and were used to quantify introgression from the Skamania stock and compare assignment proportions among local stocks. Local baseline collections included natural-origin juvenile fish (no adipose clip) from 16 sites throughout the Klickitat River (Figure 1; Supplementary Table 1). A caudal fin clip was collected nonlethally from each fish and then the fish was released back into the river. For the broadscale genetic baseline of the entire Columbia River basin, we utilized an existing baseline of samples and designated reporting groups from previous studies, as shown in Figure 3 (Hess et al. 2016a, 2022).

For the juvenile mixture samples, 2624 juveniles were collected at the Klickitat River smolt trap (river kilometer [rkm] 4.6 measuring from its confluence with the Columbia River) between 2015 and 2021 (Supplementary Table 2; Figure 1). A caudal fin clip was collected nonlethally from each fish and then released into the river. No adipose fin-clipped juveniles were detected in the smolt trap. The juvenile samples were all considered natural-origin because they were produced in a natural habitat,

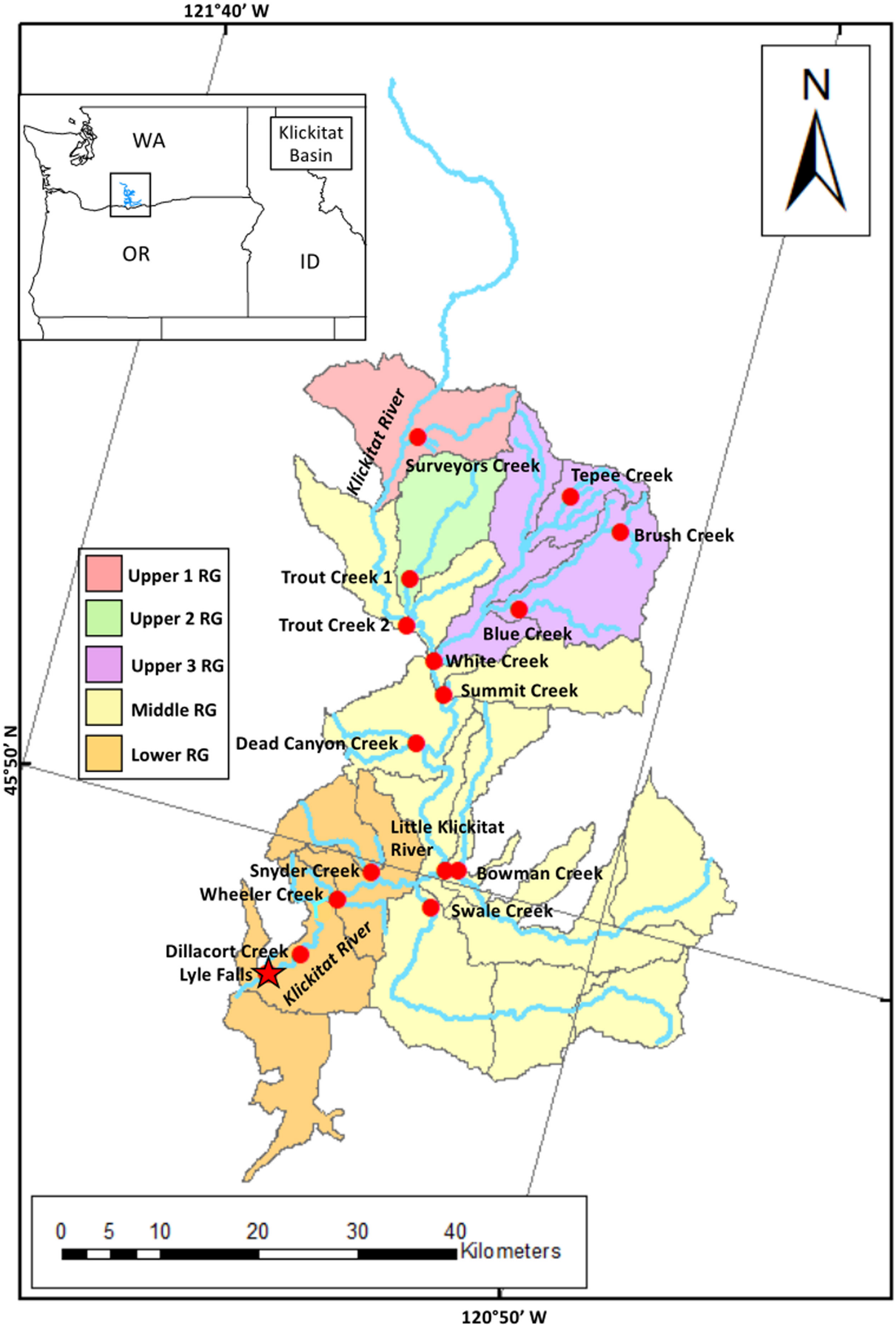

FIGURE 1 Map of the Klickitat River with local baseline collection sites (red dots; Supplementary Table 1 [available in the online version of this article]), juvenile mixtures from the screw trap, and adult mixtures from the Lyle Falls trap (red star). Five colored reporting groups (RGs) were designated based on geographic location and genetically distinct units within the subbasin.

despite whether the parents were produced from hatchery or wild spawning.

For the adult samples, we collected 3108 adult steelhead at the Lyle Falls adult trap at rkm 3.8 on the Klickitat River from 2012 to 2021 (Table 1; Figure 1). The voluntary adult trap collects an estimate of 20–30% of adult steelhead returning to the Klickitat River each year, with the majority of fish bypassing the trap and migrating over Lyle Falls (Yakama Nation Fisheries, unpublished data). Both hatchery- and natural-origin adult fish were collected in the trap, and each fish was sampled nonlethally with a caudal fin clip and then released into the river upstream of Lyle Falls. Adult samples were used to address multiple objectives including the amount of out-of-basin straying that occurs in the Klickitat River, the composition of early and late migration ecotypes of steelhead in the Klickitat River, and if Skamania stock introgression is present in natural-origin Klickitat River steelhead. Capture date in the adult trap were also the primary migration timing information used to verify the relationship with genetic markers associated with this trait. The origin of adults was initially designated based on the presence or absence of an adipose clip. Hatchery-origin steelhead typically have clipped adipose fins, but not every hatchery steelhead adipose fin gets clipped due to either hatcheries not following this procedure or occasionally missing a fish. Further analyses to identify unclipped hatchery-origin fish are described in the PBT section below.

In addition to migration date of all adult steelhead collected at the trap at Lyle Falls, a small number of additional returning adults ($n = 1758$) were identified through PIT tag recoveries after these fish were tagged previously as juveniles. The PIT tag data were collated from the PTAGIS database (Pacific States Marine Fisheries Commission 2021) from fish that received tags as juveniles within the Klickitat River at the same time as genetic samples were collected. The samples that were genotyped included individuals that were detected along their returning migrations and only fish with complete migration data were included. "Complete" migration data were defined by a fish having a detection at the Lyle Falls adult trap, the Lyle Falls Fishway (rkm 3.8, same facility as the adult trap), or the Klickitat River Floating Array (rkm 4.6). However, the PIT tag data set was not expected to reflect representative sampling across the Klickitat River steelhead runs each year. This is because very few detections ($n = 70$) were in tributaries to the Klickitat River (Snyder Creek, Swale Creek, Little Klickitat River, Summit Creek, and White Creek; Supplementary Table 4). Individual migration timing to one of the primary spawning areas in the White Creek tributary was also tracked with PIT tags, since fish detected in the lower Klickitat River near Lyle Falls may represent individuals seeking temporary refuge during their migration to other subbasins in the Columbia River (Keefer et al. 2009, 2018). Previous studies have

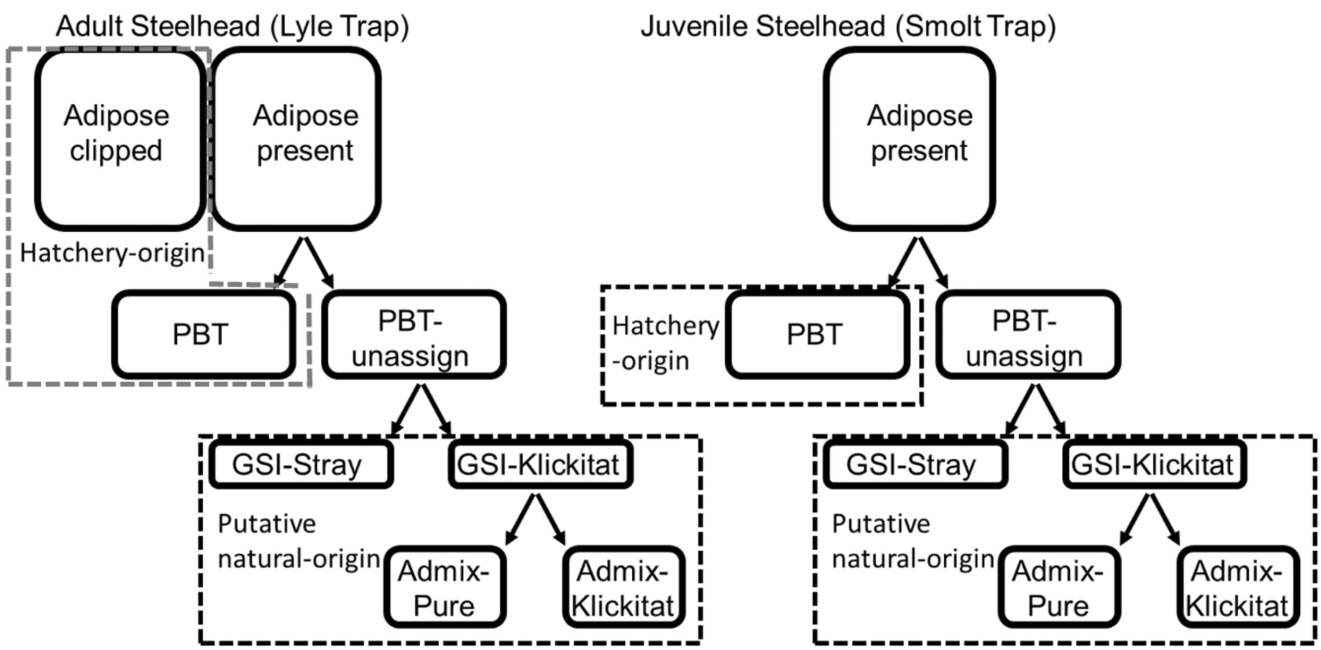

FIGURE 2 Diagram of steelhead samples collected as either juveniles or adults, and the analysis process with either parentage-based tagging (PBT) or genetic stock identification (GSI). Fish with adipose clips were identified as hatchery-origin along with any other fish that were assigned to hatchery parents with PBT. All other fish were considered natural-origin and assigned to reporting groups with GSI either at local-scale within the Klickitat River, or as strays from other major subbasins in the Columbia River.

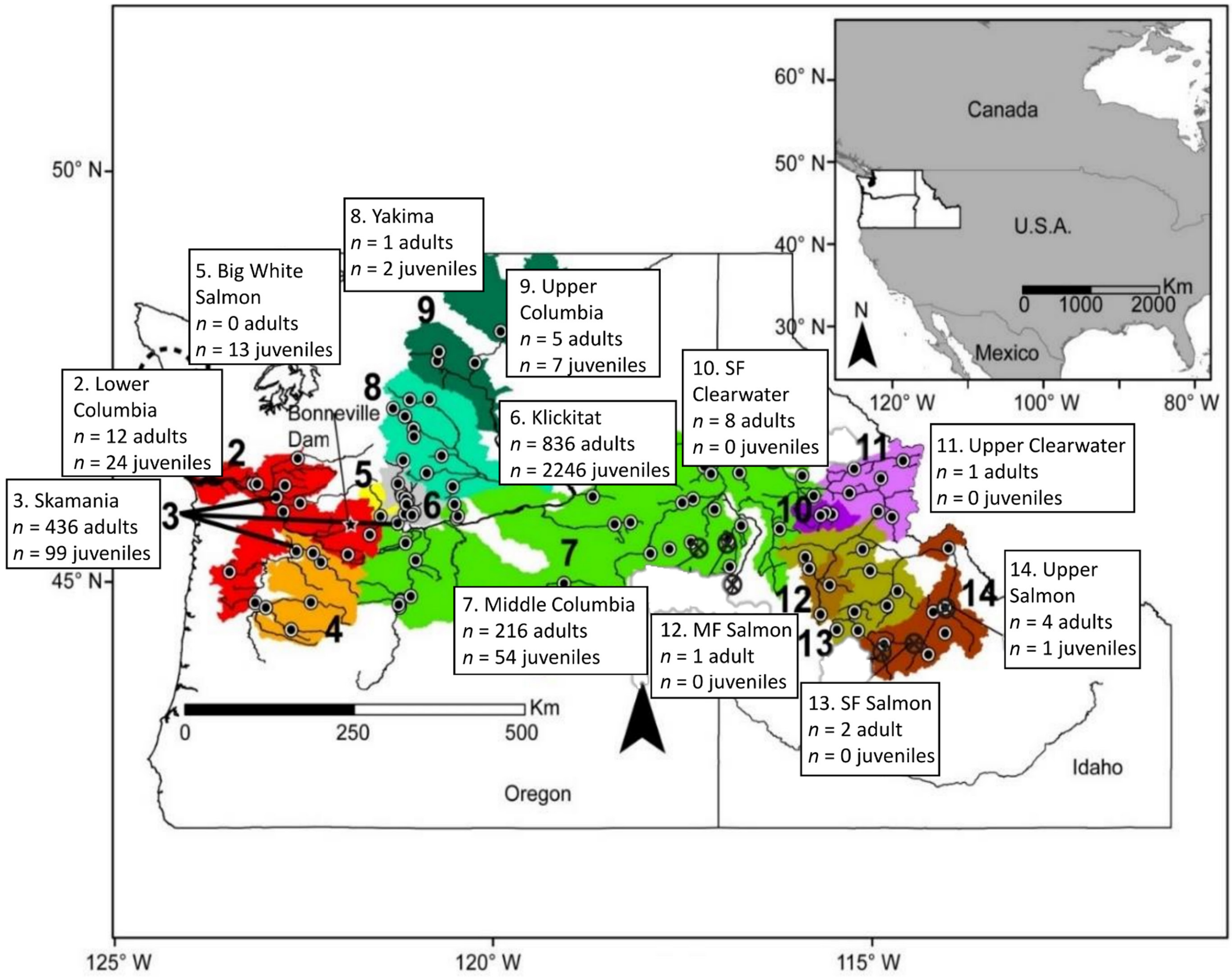

FIGURE 3 Genetic stock identification (GSI) assignments across the Columbia River basin for adult and juvenile steelhead collected from 2012 through 2021. (Adapted from Hess et al. 2022.)

indicated that the genetic markers associated with migration timing suggest association with both freshwater entry and timing of arrival to spawning grounds (Micheletti et al. 2018b; Willis et al. 2020).

Molecular methods

DNA was extracted from all fin clip tissues of (Supplementary Tables 2 and 3) hatchery- and natural-origin, adult and juvenile steelhead with the Chelex 100 method (Sigma-Aldrich, St. Louis, Missouri, USA) for the genotyping-in-thousand by the sequencing method (GT-seq) as described by Campbell et al. (2015). Standard GT-seq methods were performed in two sessions of polymerase chain reaction (PCR), the first amplified the targeted single nucleotide polymorphisms (SNPs) and the second added dual barcodes to label all individuals for further analyses. Sample concentrations were normalized and pooled into a library after barcoding. Sequencing was then done on the Illumina NextSeq 550 (San Diego, California, USA) instrument and genotypes were determined using updated scripts to account for all markers (Collins et al. 2020) beyond the original panel (Campbell et al. 2015). Samples and loci were removed from the data set if they had ≥10.0% genotypes missing. A mix of 376 putatively neutral and adaptive SNP markers were included in the GT-seq panel to genotype these samples (Collins et al. 2020). The GT-seq SNP panel included 13 *greb1L/rock1* markers associated with adult migration timing in steelhead. Only samples that were successfully genotyped at ≥90% of the SNP markers were retained for statistical analyses. To estimate the genotyping error rate across the full set of samples in the study, approximately 2% of all fish were genotyped twice to test for concordant results for the same fish.

TABLE 1 The number of *O. mykiss* samples (*n*) that were assigned to the Klickitat River for each year and life stage. The proportions of the samples that assigned to the Klickitat River are also reported for each assigned Klickitat River subbasin reporting group.

Klickitat River tributary	*n*	Upper Klickitat1 Surveyors Creek	Upper Klickitat2 Trout Creek1	Upper Klickitat3	Middle Klickitat	Lower Klickitat
Juvenile 2015	22	0.000	0.000	0.136	0.818	0.045
Juvenile 2017	18	0.000	0.000	0.278	0.611	0.111
Juvenile 2018	484	0.010	0.000	0.235	0.742	0.012
Juvenile 2019	1256	0.001	0.000	0.357	0.467	0.176
Juvenile 2021	466	0.002	0.000	0.191	0.727	0.079
Adult 2012	61	0.000	0.000	0.098	0.902	0.000
Adult 2013	34	0.000	0.000	0.088	0.912	0.000
Adult 2014	50	0.000	0.000	0.100	0.860	0.040
Adult 2015	216	0.000	0.000	0.130	0.847	0.023
Adult 2016	83	0.000	0.000	0.301	0.651	0.048
Adult 2017	21	0.000	0.000	0.143	0.857	0.000
Adult 2018	84	0.000	0.000	0.190	0.798	0.012
Adult 2019	75	0.000	0.000	0.213	0.693	0.093
Adult 2020	168	0.000	0.000	0.214	0.756	0.030
Adult 2021	44	0.000	0.000	0.250	0.728	0.023

Statistical analyses

For mixture samples of both adults and juveniles collected in the lower Klickitat River, PBT and GSI methods were used together to assess the composition of steelhead passing through the Lyle Falls adult trap and Klickitat River smolt trap (Figure 1). To assign steelhead sampled at the adult and smolt traps, PBT analysis was conducted first by comparing the genotypes of the adult and juvenile unknown steelhead to a database of previously collected steelhead with known origins as potential parental matches (PBT baseline included all steelhead hatchery stocks above Bonneville Dam from spawn years 2008 to 2019; data set ID# 20220330; www.fishgen.net). Since hatchery broodstock were sampled and genotyped each year, then offspring of the broodstock will assign to their parents with high confidence (e.g., Steele et al. 2019). However, not all broodstock were collected every year from every hatchery, and some hatchery-origin fish can go unassigned with PBT assignment methods. The database was curated annually together with hatchery broodstock samples collected by state and tribal agencies throughout the Columbia River basin to build a thorough baseline of genotyped individuals to compare unknown samples with software called SNPPIT (Anderson 2012). With this analysis approach, unknown samples are genotyped and analyzed against the steelhead baseline, and origin and age of the sampled steelhead can be determined when both parents are present in the data set. Both adult and juvenile samples from 2012 through 2021 were analyzed and assignments with PBT were considered significant when the false discovery rate (FDR), or the proportion of offspring assigned to incorrect parental pairs, was equal to or less than 0.1 and when the likelihood-of-difference (LOD) score was equal to or exceeding 14. Adult and juvenile steelhead that were not confidently assigned with the PBT method were next passed through to a broadscale GSI analysis using baseline collections distributed across the Columbia River basin (177 SNP reference baseline of 13 Columbia River reporting groups similar to those described in Hess et al. 2016a). The "broadscale" GSI analysis was conducted on all juvenile and adult samples unassigned with PBT methods with a baseline of samples organized into reporting groups to assign steelhead to their likely region of origin with gsi_sim software (Anderson et al. 2008). A GSI assignment was considered successful if it surpassed a probability threshold of 0.8.

Additionally, we were able to develop a new local-scale GSI baseline from samples collected from various tributaries to the Klickitat River to delineate fine-scale reporting groups within the Klickitat River subbasin (Figure 1). Juvenile and adult samples that were assigned to the Klickitat River based on the broadscale GSI baseline were further parsed into fine-scale reporting groups within the Klickitat River basin using this "local-scale" baseline. A leave-one-out cross-validation test was conducted with Rubias software (Anderson et al. 2008) on the new local-scale GSI baseline to test the accuracy of assignments of the reference samples for this baseline, which have known origins. Each iteration of the leave-one-out test consisted of removal of an individual from the baseline and assignment of that individual to the baseline with all other individuals remaining to avoid self-assignment; this step was repeated for every individual in the baseline. A principal component analysis (PCA) of allele frequencies with 237 putatively neutral markers of genotyped Klickitat *O. mykiss* was conducted with the adegenet R package (Jombart 2008) to further discern differentiation between GSI reporting groups within the Klickitat River basin.

To assess the extent of Skamania Hatchery genetic introgression, we used the putatively neutral markers ($n=237$) without linkage disequilibrium (LD; genepop R package [Rousset 2008]) to observe the population structure of steelhead assigned to the Klickitat River with either the PBT or GSI methods (Collins et al. 2020). We used a PCA of allele frequencies of the putatively neutral markers to compare steelhead genotyped from the Skamania Hatchery to the samples assigned to the Klickitat River. Subsequently, a discriminant analysis of principal components (DAPC) was administered with the R package adegenet 2.1.0 to identify maximum genetic variation between genetic groups as genetic variation within groups is minimized (Jombart 2008). The DAPC analysis also examines the genetic similarity of ancestral groups (K) with successive K-means and ran for 25 iterations of $K=1$ through $K=10$. The Bayesian information criterion (BIC) of each K value was averaged and scaled according to the standard deviation, and the final ΔK value was selected to provide a variable for the most probable amount of ancestral populations (Evanno et al. 2005). These analyses were completed in tandem with an admixture analysis conducted with sparse nonnegative matrix factorization (snmf) methodology with the LEA 2.0 R package to assess the population structure (Frichot and François 2015). Individuals assigned to the Skamania Hatchery were included as a Skamania Hatchery reference population and Sawtooth Hatchery steelhead broodstock collected in 2019 were included as a reference population for Snake River steelhead. Mixture samples were assumed to be "pure" hatchery-origin fish (exhibiting minimal introgression) if they clustered with the Skamania Hatchery or Snake River reference populations at ≥0.90 probability with the snmf analysis or were adipose-clipped. These "pure" fish were quantified but removed as a filter to enhance the ability to describe levels of introgression by targeting the most introgressed individuals in downstream analyses.

Further, steelhead samples were categorized into early and late ecotypes based on SNPs associated with migration timing. Homozygous genotypes consisted of alleles representative of early and late migration ecotypes and were determined based on previous studies (Hess et al. 2016b; Micheletti et al. 2018b; Collins et al. 2020). A heterozygous genotype of these alleles was defined as "intermediate." The genetic markers examined were located on chromosome 28 in the gene region containing the *greb1L* gene, *rock1* gene, and intergenic region between *greb1L* and *rock1*. Migration timing of returning adults was evaluated based on the date of passage at the trap located in the lower Klickitat River at Lyle Falls.

RESULTS

Steelhead productivity in the Klickitat River subbasin

Between 2012 and 2021, a total of 4816 out of 5732 (84.0% average genotyping success) samples were successfully genotyped from the Klickitat River. For each of the mixture analyses, 2450 (93.4% average genotyping success) of the juvenile samples were successfully genotyped from the smolt trap, and 2366 adults (70.5% average genotyping success) from the Lyle Falls trap were successfully genotyped in 1849 adipose-clipped adults; 1259 were adipose-present adults (Supplementary Tables 2 and 3).

For the juvenile mixture collections, none of the samples assigned with PBT, as expected, since they were natural-origin fish. A total of 2450 juveniles were assigned with the GSI method using the broadscale baseline for the entire Columbia River basin and had a large proportion of assignments to the Klickitat reporting group (91.7%; Supplementary Table 2; Figure 1). For the adult mixture samples, a total of 701 adipose-clipped adults and 143 adipose-present adults were assigned across the Columbia River basin with the PBT method and the majority of these (681) were assigned to the Skamania Hatchery (Table 2; Supplementary Tables 2 and 3). Additionally, 1523 adult samples were assigned with the GSI method using the broadscale baseline and the majority (836; 54.9%) were assigned to the Klickitat River reporting group (Figure 1; Supplementary Tables 2 and 3).

TABLE 2 Steelhead parentage-based tagging (PBT) assignments for samples collected at the Lyle adult trap between 2012 and 2021.

Steelhead hatcheries	Number of adults assigned
Skamania Hatchery	683
Round Butte Fish Hatchery	2
Lyons Ferry Fish Hatchery	15
Little Sheep Creek Hatchery	1
Oxbow Fish Hatchery	20
Dworshak National Fish Hatchery	20
Sawtooth Fish Hatchery	3
Pahsimeroi Fish Hatchery	104
Total	848

The local-scale GSI baseline contained five reporting groups for assignment of mixture samples within the Klickitat River. Leave-one-out test results were reported as the proportion of individuals of each baseline population that were assigned to the correct reporting group (Supplementary Table 1). The “Middle Klickitat” reporting group had the highest self-assignment rate with 91% of tributary individuals assigned to the correct reporting group and the “Lower Klickitat” reporting group had the lowest, at 40%. The PCA of the individuals with known sampling locations used in the leave-one-out analysis clustered Klickitat River tributaries in accordance with the local-scale baseline (Figure 4). This local-scale GSI baseline allowed us to observe the distribution of steelhead production across tributaries in the Klickitat River subbasin. Of the juvenile and adult *O. mykiss* individuals assigned to the Klickitat River reporting group with the Columbia River baseline, the majority assigned to the “Middle Klickitat” reporting group (juvenile mean = 72.8%; adult mean = 87.3%), which includes samples from the lower Trout Creek, Summit Creek, Dead Canyon Creek, Bowman Creek, Little Klickitat River, and Swale Creek (Table 1; Figure 1). The “Upper Klickitat 3” reporting group had the next largest percentage of assignments (juvenile mean = 23.7%; adult mean = 11.4%), followed by the “Lower Klickitat” (juvenile mean = 3.4%; adult mean = 1.3%; Table 1). The other two reporting groups in the upstream reaches of the Klickitat River had nearly zero assignments, with the exception of a few fish assigned to Surveyors Creek in the Upper Klickitat River subbasin (Table 1). Thus, steelhead production was not evenly distributed throughout the Klickitat River tributaries for any life stage or year. The “Middle Klickitat” reporting group had the highest average proportion of assignments but likely included misassigned fish from other tributaries, especially those from the “Lower Klickitat.”

Straying and introgression from hatchery stocks of steelhead

We identified hatchery-origin steelhead in the Klickitat River using physical marks (adipose clips = 389), PBT results (fish that were assigned to the PBT baseline = 848), and GSI results (fish that were assigned with a high probability to the reference hatchery stocks = 127). Analyses with PBT enabled adults to be assigned to their hatchery of origin and identified a total of 683 Skamania steelhead and 165 from outside the Klickitat River and (Table 2). Adult hatchery steelhead strayed into the Klickitat River from the following hatcheries: Pahsimeroi (n = 104; 12.3%), Oxbow (n = 20; 2.4%), Dworshak (n = 20; 2.4%),

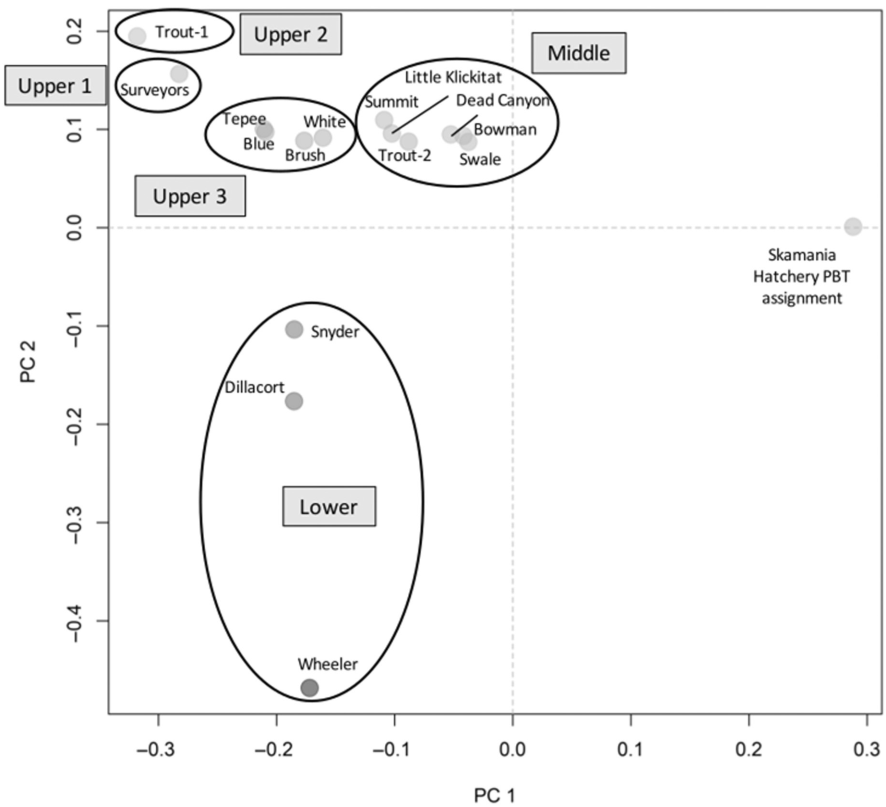

FIGURE 4 Principal component analysis (PCA) of collections of *O. mykiss* from tributaries throughout the Klickitat River subbasin. Skamania Hatchery steelhead are included as a reference for the primary hatchery stock released in this system.

Lyons Ferry ($n=15$; 1.8%), Sawtooth ($n=3$; 0.4%), Round Butte ($n=2$; 0.2%), and Little Sheep Creek ($n=1$; 0.1%) hatcheries. We quantified the "pure" hatchery-origin fish from all adult and juvenile samples analyzed with smnf that exhibited minimal introgression and clustered with the Skamania Hatchery or Snake River reference populations at ≥0.90 probability. Before running the admixture analysis of natural-origin fish, individuals that clustered with the Skamania Hatchery samples (4 adults, 53 juveniles) and clustered with individuals collected from hatcheries in the Snake River (13 adults, 57 juveniles) and 389 adipose-clipped adults were removed.

Introgression in the remaining natural-origin fish (739 adults, 3601 juveniles) was analyzed with DAPC analyses to estimate the proportion of ancestry to various groups (admixture coefficient). The DAPC with neutral markers grouped steelhead into two clusters ($\Delta K=2$) when focused only on natural-origin samples within the Klickitat River: the first cluster predominately contained samples assigned to the Skamania Hatchery and assignments to the Klickitat reporting groups formed the second cluster (Supplementary Figure 1 [available in the online version of this article]). Further analyses with neutral markers revealed a greater number of clusters with more subdivisions within the natural-origin Klickitat River samples based on the cross-entropy values from the snmf analysis. The admixture coefficients were plotted for $K=5$ with the LEA R package to visualize the genetic mixing of samples assigned to the Skamania Hatchery; the upper, lower, and middle tributaries to the Klickitat River; and Snake River reference samples (Figure 5; Supplementary Figure 3). The admixture analysis divided the Klickitat River into similar groups as the PCA and each color in the plot indicates the proportion of each ancestral coefficient for every individual (Figure 5; Supplementary Figure 3). Analyses also revealed that the samples that were assigned to the Skamania Hatchery were distinct and that a degree of introgression was present in all Klickitat River reporting groups, but the Middle and Lower Klickitat River reporting groups contained the highest rate of introgression from the Skamania Hatchery (red) (Figure 5; Supplementary Figure 2). Admixture was also apparent from the orange ancestral coefficient, which could be attributed to either the Snake River reference collection or the outside Klickitat River collections (Figure 5; Supplementary Figure 3).

For our collection of adult adipose-present steelhead in the Klickitat River ($n=1153$), there were 143 that were PBT assigned (i.e., adipose-present hatchery-origin fish). Of the remaining 1010 adipose-present adults that were not assigned with PBT (i.e., putatively natural-origin), there were 780 that were assigned to the Klickitat reporting group and 230 that were assigned to reporting groups outside the Klickitat River. Of those putatively natural-origin GSI-assigned adults, based on the admixture results, there were four "pure" Skamania fish and 13 "pure" fish from outside the Klickitat River. Adults with clipped adipose fins that failed to be assigned to Skamania with PBT methods, but were considered "pure" Skamania fish, were likely due to PBT tag rates <100.0% of Skamania Hatchery broodstock (Supplementary Table 6). Of the remaining 763 steelhead that were putatively natural-origin from inside the Klickitat River (excluding all GSI-assigned strays and admixture pure strays), we estimated that an average of 11.3% introgression from the Skamania stock and 23.0% introgression from Snake River/Outside Klickitat (Supplementary Figure 2 and See Figure 2 diagram of these analytical categories of introgression). Similarly, of the 2450 juvenile steelhead successfully genotyped (i.e., putatively natural-origin Klickitat River steelhead), there were 202 fish (8.2%) that were assigned to the GSI reporting groups outside of the Klickitat River. This was higher than the expected rate of the misassignment rate from outside reporting groups, which suggests most of these were offspring of adult steelhead that strayed into the Klickitat River. For the remaining 2248 juveniles that were putatively natural-origin fish from Klickitat River spawners, based on admixture results there were still 53 "pure" Skamania fish and 57 "pure" fish that clustered with stocks outside the Klickitat River based on genetic similarity. Similar to the individuals identified as of "outside" origin from the broad scale GSI assignments, these "pure" individuals from outside stocks may represent naturally produced offspring of stray parents into the system. Of the remaining 2138 fish that were putative natural-origin fish from Klickitat River spawners (i.e., excluding all GSI-assigned and admixture "pure" fish), we estimated an average of 8.7% introgression from Skamania and 21.3% introgression from Snake River/Outside Klickitat (Supplementary Figure 2).

Patterns of steelhead migration timing and genetic variation

Individual steelhead migration timing was primarily based on the date of passage at the trap at Lyle Falls, and was compared to migration timing-associated genotypes of samples collected between 2013 and 2021. Fish with each of the migration genotypes were detected throughout the year (Figure 6), but clustered around dates that corresponded to their expected migration timing based on the presence of early vs. late alleles (Figure 6). The early migration-associated genotype was most common in samples collected from the Lyle Falls adult trap ($n=1159$; 68.3%) and were detected every month, despite the highest concentration in the summer. The median detection date of samples with the early migration genotype was June 17 and one standard deviation from the average detection date ranged

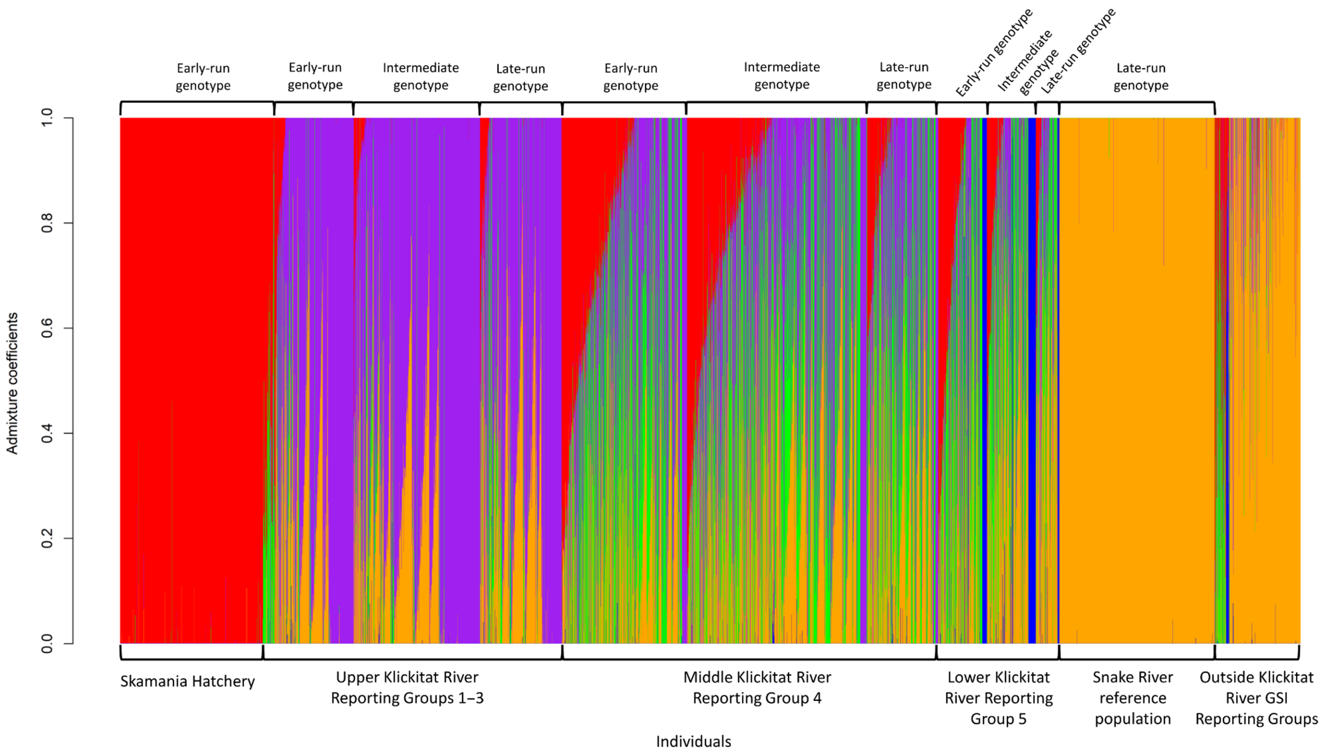

FIGURE 5 Admixture plots of natural-origin juvenile steelhead mixture samples from the Klickitat River with individuals sorted by geographic location (bottom) and migration ecotype (top). Admixture results for neutral markers ($\Delta K=5$) with individuals assigned to various fine-scale GSI reporting groups (Skamania Hatchery = red, Upper Klickitat = purple, Middle Klickitat = green, Lower Klickitat = blue, and a Snake River reference population = orange).

from May 3 through July 31 (Table 3). Steelhead with the heterozygous migration timing-associated genotype were less common ($n=187$; 11.0%) and occurred at the Lyle Falls adult trap in all months with a median detection date of July 14 (one standard deviation from the average detection date ranged from May 1 through September 28; Table 3). There were fewer steelhead with the heterozygous migration timing-associated genotype than expected from tests of Hardy–Weinberg equilibrium (Supplementary Table 7). Steelhead with the late migration genotype were detected at the Lyle Falls adult trap ($n=350$; 20.6%) and occurred in all months except June. The median detection date for samples with the late migration genotype was April 1 and one standard deviation from the average detection date ranged from January 19 through July 23 (Table 3).

Genotypes associated with migration timing (*greb1L/rock1* markers) were assessed for both natural- and hatchery-origin adult steelhead, but also for juvenile steelhead collected with unknown return timing to evaluate the frequency of migration-associated alleles for steelhead in the Klickitat River subbasin (Supplementary Table 5). Natural-origin steelhead had nearly equal allele frequencies of the early summer-run (0.55) and late winter-run (0.45) migration timing-associated alleles. All adult steelhead collected at Lyle Falls that were assigned to the Skamania Hatchery with PBT were assessed at the alleles associated with migration timing and consisted mostly of early (0.92) and fewer late (0.08) alleles (Supplementary Table 5). The samples in the admixture plot (Figure 5; Supplementary Figure 3) that clustered with the Skamania Hatchery consisted of individuals with a higher frequency of alleles associated with early migration, and the Snake River reference population consisted of individuals with a higher frequency of alleles associated with late migration.

DISCUSSION

In this study we implemented genetic monitoring tools in conjunction with extensive field surveys of steelhead in the Klickitat River to provide insight into productivity, straying, and migration timing of steelhead in this subbasin. The results indicated that productivity of steelhead in the middle Klickitat River tributaries to be particularly productive relative to nearby tributary systems. Straying was detected from outside stocks for both hatchery- and natural-origin stocks with PBT and GSI assignments, and introgression from stocks outside of the Klickitat River was estimated with admixture analyses. We detected the most introgression from the Skamania Hatchery stocks within fish from the middle Klickitat River and the early-run-associated genotype. Additionally, patterns of adult

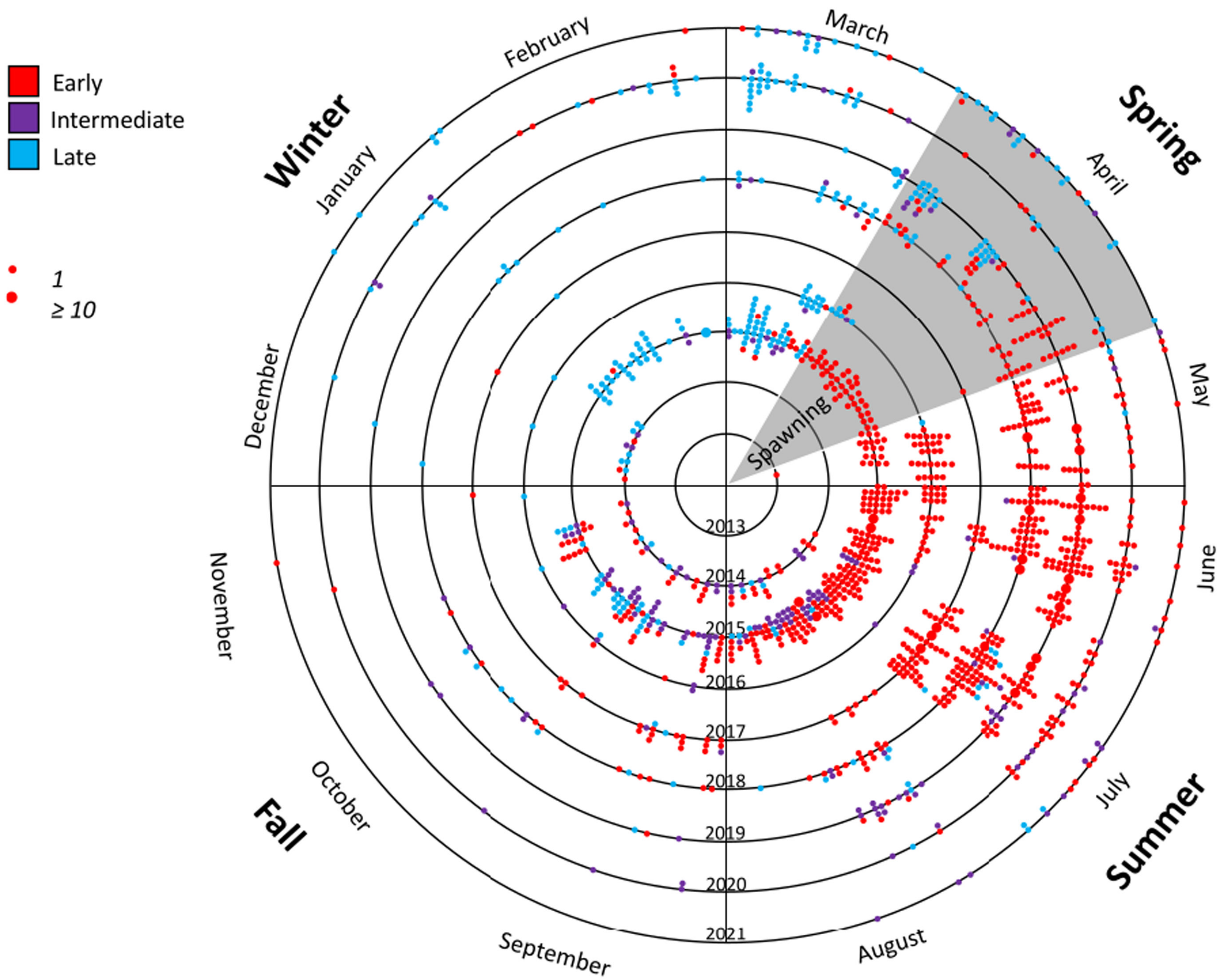

FIGURE 6 Seasonal migration timing for individual adult steelhead captured at the Lyle Falls trap in the lower Klickitat River between 2013 and 2021. Black rings of the circle represent steelhead migrations for each sample year. The approximate spawning season in upstream tributaries is shown in the gray-shaded piece of spring, but migration timing of steelhead is shown as observed earlier at the trap located downstream at Lyle Falls. Each point is positioned according to the date each adult steelhead returned to the Klickitat River, with colors to indicate the genotype (homozygous early, heterozygous/intermediate, and homozygous late). The size of each dot reflects the number of samples for a given date.

TABLE 3 Steelhead migration timing for all individuals collected from the Klickitat River and genotyped at loci associated with migration timing (early, late, or heterozygous genotypes).

Migration timing genotype	# of individuals	% of total samples	Months detected	Median detection date	One standard deviation detection dates
Early	1118	68.8	all	Jun 23	May 9–Aug 7
Heterozygous	183	11.2	all	Aug 4	May 21–Oct 18
Late	325	20.0	all, except Jun	Mar 28	Dec 26–Jun 30

migration timing at the Lyle Falls trap were largely consistent with migration-associated alleles, and we found that early and late migration alleles were more evenly balanced in natural-origin samples than in hatchery-origin samples. This study provides an improved understanding of steelhead tributary productivity, straying, and hatchery- and natural-origin introgression in the Klickitat River population, which contributes to the toolkit for managers of an Endangered Species Act threatened steelhead population.

Steelhead productivity in the Klickitat River subbasin

Natural-origin productivity of steelhead in the Klickitat River was unevenly distributed and predominantly comprised of fish from the middle Klickitat River tributaries, despite these spawning grounds representing less than a third of the total available spawning ground habitat of the Klickitat River subbasin. Previous studies (Narum et al. 2006, 2008) collected samples from similar locations and within each of the subgroups (Upper, Middle, Lower) of the Klickitat River, but the previous studies did not assign samples of unknown origins within subgroups of the Klickitat River. The approach of genetic monitoring with a local GSI baseline provided estimates of productivity to various sections of this subbasin, with the highest productivity of steelhead occurring in the middle and upper portions of the Klickitat River, with uncertain productivity in the lower Klickitat and nearly zero in the most upstream reaches of the subbasin.

While most fish were assigned with GSI to the Middle Klickitat reporting group, there may have been several misassigned fish to this group that actually were produced in tributaries of the Lower Klickitat reporting group or White Creek. Misassignments with GSI analyses are common when groups are not highly distinct from one another (Beacham et al. 2006), and this was evident from the leave-one-out tests where a portion of fish from other tributaries (i.e., Lower Klickitat) misassigned to the Middle Klickitat reporting group. Further, the asymmetry of misassignments to the Middle Klickitat suggests that productivity estimates are biased high for this group. Future studies may be able to address this type of bias in estimates by including sibship analyses (e.g., Ruzzante et al. 2019). Additionally, collection locations for mixture samples in the Klickitat River could be expanded in the future to provide replication and further representation of steelhead produced in this system.

The adult, adipose-clipped, hatchery-origin steelhead in the Klickitat River were found to be mostly from the Skamania Hatchery, as expected given the high numbers of juveniles that are released in the subbasin for harvest opportunities of returning adults (National Marine Fisheries Service 2017). However, adipose-clipped, and adipose-present hatchery-origin strays were detected from hatcheries operating outside the Klickitat River, most of them from Snake River hatcheries that are the predominant source of all hatchery-origin steelhead above Bonneville Dam (Hess et al. 2016a; Steele et al. 2019; DeHart 2020). For the adult steelhead, both adipose-present hatchery-origin steelhead (i.e., adipose-present, PBT-assigned fish) and adipose-present putatively natural-origin steelhead were observed in spawning reaches of the Klickitat River (Zendt et al. 2021). Adipose-present steelhead that were assigned to a hatchery with PBT were first-generation hatchery-origin fish that were not clipped or originated from a hatchery that does not clip adipose fins, which has been demonstrated for steelhead hatchery programs in the region (e.g., Hargrove et al. 2021).

Straying and introgression from hatchery stocks of steelhead

Hatchery steelhead strayed into the Klickitat River from multiple programs in the interior Columbia River basin, with the largest proportion from the Pahsimeroi Hatchery ($n=104$; 12.3%). The Pahsimeroi Hatchery is one of the furthest inland hatcheries for the strays collected at the Lyle Falls adult trap and there is the possibility that these steelhead used the Klickitat River as a thermal refuge on their way further inland, as previously documented (Keefer et al. 2009, 2018). However, other factors, such as production size from each broodstock program, may contribute to relative estimates of strays detected in this study. Strays from all other hatcheries were less common, between 1 and 20 individuals collected at the Klickitat River adult trap. Differing stray rates observed among hatchery programs may also be due to variation in juvenile rearing, acclimation, and release practices that may affect homing ability (e.g., Paquet et al. 2011; Keefer and Caudill 2014).

We observed that the influence of introgression on natural-origin Klickitat River steelhead from summer-run hatchery fish was not evenly distributed geographically, or among migration ecotypes throughout the subbasin. Admixture analysis also revealed a degree of introgression of the Skamania stock in all reporting groups, particularly in the summer ecotype of the Middle Klickitat reporting group. Despite evidence for introgression, Narum et al. (2006) found that Skamania stock steelhead are genetically distinguishable from native stocks, and this was further supported by the PCA and admixture analyses in this study (strong differentiation of Skamania Hatchery-assigned samples from the other Klickitat River samples).

Assignments from PBT also determined whether an individual collected at the Lyle Falls adult trap or the screw trap was natural-origin or hatchery-origin; however, this method did not account for PBT tag rates. Thus, in years when Skamania stock had low (2016) or unknown (2012–2013) PBT tag rates and the individual did not assign with PBT, we were not able to differentiate between a true natural-origin steelhead or one that was not assigned to the Skamania Hatchery due to low PBT tag rates. Future applications may be able to incorporate PBT tag rates to improve estimates further (e.g., Delomas and Hess 2021).

Patterns of steelhead migration timing and genetic variation

Steelhead adults returning to the main-stem Klickitat River provided insights into steelhead migration and spawn timing related to the respective genotypes associated with migration timing. Steelhead with genotypes associated with early migrations were detected returning to the main-stem river in the spring and summer, while steelhead with genotypes associated with intermediate migration timing were detected returning in the late summer and early fall, and steelhead with late genotypes arrived in winter and spring shortly before expected spawning. However, there were outliers present for all three genotypes with unexpected migration timing. In general, these patterns align with what would be expected from genotypes that are associated with migration timings, which has been demonstrated by previous studies (Hess et al. 2016b; Prince et al. 2017; Micheletti et al. 2018b; Collins et al. 2020; Willis et al. 2020). The lower-than-expected frequency of heterozygous fish, as confirmed with a Hardy–Weinberg equilibrium analysis of loci associated with migration timing, suggests differences in spawn timing or other isolating mechanisms between fish that were homozygous for early versus late migration alleles.

Overall patterns of migration timing differed between hatchery- and natural-origin steelhead in the Klickitat River due to the Skamania Hatchery stock that has been selected for early summer-run fish (Ayerst 1977) and represent the vast majority of early-returning steelhead in the Columbia Basin (Hess et al. 2016a). Hatchery-origin steelhead collected at Lyle Falls between 2012 and 2021 were predominantly from Skamania stock that mostly consisted of alleles associated with early (0.92) migration timing, whereas collections of natural-origin steelhead are more evenly split between the late (0.44) and early (0.56) alleles associated with migration timing. These results were consistent with previous analyses (Collins et al. 2020) that showed Klickitat River reference collections of natural-origin samples had a similar proportion of early alleles (0.64) and Skamania Hatchery stock samples consisted only of early alleles (1.00). In contrast, stray steelhead from Snake River hatcheries or other sources outside of the Klickitat River primarily influenced the Lower Klickitat River reporting group and contained a high proportion of late alleles. These different sources of strays may contribute both early and late alleles if they reproduce successfully in nature and produce natural-origin Klickitat River steelhead. Previous studies have demonstrated that Skamania Hatchery steelhead have low reproductive success (Leider et al. 1990; Kostow et al. 2003) and have provided evidence for reproductive isolation from Klickitat River natural-origin steelhead (Narum et al. 2006). However, reproductive success of these different hatchery stocks is uncertain in the Klickitat River and does not appear to have greatly skewed the frequency of early versus late migration alleles in the natural population.

Management implications

Steelhead in the Klickitat River have retained genetic diversity due to gene flow among neighboring populations, but also between the coastal and inland steelhead lineages that co-occur in this intermediate geographic location near the crest of the Cascade Mountain range. High genetic diversity in this region was expected, based on previous studies of steelhead (e.g., Blankenship et al. 2011) and we suggest more nuance in how introgression results are interpreted. Uncertainty remains regarding the level of introgression that adversely impacts fitness and the trade-offs of dispersal among populations (Keefer and Caudill 2014). Over time, multiple sources of introgression may have influenced Klickitat River steelhead populations, including both natural- and hatchery-origin strays.

Analyses revealed genetically mixed Klickitat River populations that were distinguishable from both the Skamania stock and Snake River reference samples. Interestingly, most natural-origin strays clustered with the same ancestral coefficient as the Snake River reference group along with any other stocks outside the Klickitat River with the admixture analysis, while a few natural-origin strays assigned to coastal GSI reporting groups with the broadscale GSI analysis. This suggests a broad influence of dispersal on maintenance of diversity in steelhead in the Klickitat River. Levels of introgression appear higher from Snake River stocks and other stocks outside the Klickitat River than the Skamania stock, which is consistent with telemetry data that revealed Snake River fish remain in the Klickitat River subbasin over periods in known locations of spawning activity (Zendt 2018). Overall, introgression as a result of natural-origin dispersal is a natural phenomenon (Keefer et al. 2018), but it is important to continue to monitor for signs of human influence, such as exceptionally high stray rates.

Under its Endangered Species Act threatened status as part of the Middle Columbia River ESU, hatchery juvenile releases and monitoring activities are covered under a biological opinion (National Marine Fisheries Service 2017). This biological opinion calls for the percent of hatchery-origin spawners (pHOS) on natural spawning grounds of 5.0% or less, but pHOS is not easily estimated on the Klickitat River due to a low number of steelhead carcasses recovered most years during spawner surveys that co-occur with high spring flows. However, these pHOS measures may not directly influence productivity of natural-origin steelhead (Courter et al. 2022) and other approaches to evaluating the effects of hatchery programs may be necessary. While the National Marine Fisheries Service gene flow metric may not be directly comparable to the results presented here, the available measures from this study indicate a generally low level of gene flow between Skamania Hatchery steelhead and natural-origin Klickitat steelhead populations. We suggest that this multiyear data set could be useful as a reference to compare future introgression analyses in the Klickitat River to monitor for sudden changes that may indicate a need for alternative management actions.

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CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships.

DATA AVAILABILITY STATEMENT

Genotype data for the local Klickitat River baseline and mixtures are available on Dryad (https://doi.org/10.5061/dryad.rfj6q57df).

ETHICS STATEMENT

The animal study was reviewed and approved by Monitoring Resources; Methods (monitoringresources.org).

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SUPPORTING INFORMATION

Additional supplemental information can be found online in the Supporting Information section at the end of this article.