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Whole genome resequencing reveals genomic regions associated with thermal adaptation in redband trout

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Abstract

Adaptation to local environments involves evolution of ecologically important traits and underlying physiological processes. Here, we used low coverage whole-genome resequencing (lcWGR) on individuals to identify genome regions involved in thermal adaptation in wild redband trout *Oncorhynchus mykiss gairdneri*, a subspecies of rainbow trout that inhabits ecosystems ranging from cold montane forests to high elevation deserts. This study includes allele frequency-based analyses for selective sweeps among populations, followed by multiple association tests for specific sets of phenotypes measured under thermal stress (acute and chronic survival/mortality; high or low cardiac performance groups). Depending on the groups in each set of analyses, sequencing reads covered 43%–75% of the genome at ≥15× and each analysis included millions of SNPs across the genome. In tests for selective sweeps among populations, a total of six chromosomal regions were significant. The further association tests for specific phenotypes revealed that the region on chromosome 4 was consistently the most significant and contains the *cerk* gene (ceramide kinase). This study provides insight into a potential genetic mechanism of local thermal adaptation and suggests *cerk* may be an important candidate gene. However, further validation of this *cerk* gene is necessary to determine if the association with cardiac performance results in a functional role to influence thermal performance when exposed to high water temperatures and hypoxic conditions.

KEYWORDS

cardiac function, ceramide 1-phosphate, rainbow trout, sphingolipids, thermal tolerance, whole-genome resequencing

1 | INTRODUCTION

A goal of studying local adaptation is to identify the underlying genetic variation (i.e., genes and nucleotide polymorphisms) in natural populations. However, it is a challenging task since in most natural ecosystems, multiple environmental factors work together in complex ways to alter selection on a combination of traits and influence overall fitness. For example, water temperature has a pervasive effect on development, swimming performance, reproduction, and survival of ectothermic aquatic species (Beacham & Murray, 1989; Eliason et al., 2011; Narum et al., 2013; Pankhurst & King, 2010). Therefore, although thermal adaptation and related phenotypic traits are widely studied, primary genetic mechanisms remain unclear, especially in non-model aquatic species.

Thermal adaptation is expected to be complex in aquatic ectotherms given the wide range of biological processes and underlying mechanisms that are influenced by temperature and dissolved oxygen. A leading hypothesis on thermal adaptation called oxygen- and capacity-limited thermal tolerance proposes that temperature-dependent performance is limited by oxygen delivery for

energy production (Pörtner & Farrell, 2008). It hypothesizes that, in warming scenarios, metabolic activities in ectothermic animals increase exponentially with temperature, but it has a ceiling set by the oxygen transport capability of cardiorespiratory system. Therefore, cardiorespiratory system might be a target of selection in warming environment for better aerobic performance in activities such as swimming, growth, reproduction, and predation (Farrell, 2009). This hypothesis has been repeatedly supported in salmonids species based on findings that heart has a central role in thermal adaptation (Chen et al., 2018a; Eliason et al., 2011). This suggests that there is a quantitative genetic basis related to cardiac performance that may help understanding of the physiological and molecular basis of thermal adaptation in aquatic ectotherms. However, a common limitation in many genomic studies is insufficient marker density and therefore lack of necessary resolution to identify candidate genes for species with rapid linkage decay (Hoban et al., 2016; Lowry et al., 2017). One promising approach to overcome this limitation is low coverage whole-genome resequencing (lcWGR; Fuentes-Pardo & Ruzzante, 2017; Therkildsen & Palumbi, 2017), which provides high marker density with a good balance between genome coverage and cost depending on study objectives. The lcWGR approach has been successfully used to identify candidate genes for local adaptation and complex life-history traits (e.g., Therkildsen et al., 2019).

Redband trout *O. mykiss gairdneri* is a subspecies of rainbow trout *O. mykiss* native to the interior Pacific Northwest of North America. Redband trout has a wide geographical distribution from high elevation montane forests to lower-elevation sagebrush deserts. In many habitats, it may be the only fish species and thus is critical to the local ecosystem. Habitats of redband trout have been influenced by anthropogenic activities such as logging, cattle grazing, and water management (Meyer et al., 2010). Additionally, climate change is a major concern in the 21st century and could cause local extirpation of redband trout given low habitat connectivity, altered ecosystems (Isaak & Rieman, 2013), and limited gene flow observed among populations (Kozfkay et al., 2011; Narum et al., 2010) that hinders the infusion of adaptive alleles and increases the risk of inbreeding depression. Although desert redband trout have shown abilities to thrive in some of the warmest habitats for salmonids, their capability of surviving future rapid warming scenarios is questionable for populations with limited adaptive potential.

Thermal adaptation in *O. mykiss* has been studied by comparing individuals from populations with different locally adapted phenotypes (Chen et al., 2018a,2018b; Narum et al., 2013). This approach requires knowledge of specific phenotypes that are adapted to local thermal regimes, but only a few studies have attempted to identify quantitative trait loci (QTLs) in natural populations of *O. mykiss* for upper thermal tolerance (Narum et al., 2013). Additional studies in redband trout have identified intraspecific divergence in critical thermal maximum, cardiac performance, metabolic rate and gene expression between desert and montane populations (Chen et al., 2018a,2018b) but primary candidate genes remain elusive with reduced representation sequencing approaches (Chen et al., 2018a,2018b; Narum et al., 2013).

In this study, we performed lcWGR to estimate allele frequencies from high-density SNP markers to test for selective sweeps among ecologically divergent populations and also for association with specific phenotypes measured under thermal stress (acute and chronic survival/mortality; high or low cardiac performance groups). This study included samples from previous population genomics studies that were limited by marker density but had well defined phenotypes related to thermal stress (Chen et al., 2018a,2018b; Narum et al., 2013). Our sequencing strategy was lcWGR of individually barcoded samples with a target coverage of approximately 0.5–1× per individual that were then arranged into groups for analyses of allele frequencies of SNPs across the genome. Sequencing at this low coverage limited the ability to call genotypes for barcoded individuals, but the advantage of this strategy compared to standard Pool-seq (i.e., sequencing on DNA pools of many individuals that are not barcoded; Schlötterer et al., 2014) is the flexibility to form different 'pools' in the bioinformatics steps because reads are barcoded for each individual. For example, samples could be pooled (or grouped) according to population or phenotype after normalizing sequence reads per individual. This approach was intended to initially identify general signals of genomic divergence across populations, but then narrow results to identify candidate genes for phenotypes specifically related to thermal adaptation as opposed to other locally adapted traits. Development of candidate markers for further validation are expected to be applied to address questions regarding the adaptive capacity of this species under scenarios of climate change.

2 | MATERIALS AND METHODS

2.1 | Study populations

A total of 286 redband trout from three natural populations and a lab-produced hybrid strain were used in this study (Table 1). The three natural populations were ecologically divergent (Figure 1a): (i) Little Jacks Creek, a tributary of Bruneau River in high elevation desert (referred to as DESERT; $n = 99$); (ii) Keithley Creek, a tributary of Weiser River in the cool montane forest (MONTCOOL; $n = 96$); and (iii) Fawn Creek, a tributary of Payette River in the cold montane forest (MONTCOLD; $n = 49$). Production of first-generation hybrid strains were attempted in the lab but only one strain was successfully produced from crossing DESERT and MONTCOOL (hybrids were referred to as F1_DEST × MTCOOL; $n = 42$). These redband trout populations also represent the greatest range of ecotypic and phenotypic variation that has been observed. These fish were originally used for two research projects that investigated the intraspecific variation in thermal performance in redband trout (Table 1): one was on acute and chronic thermal tolerance ($n = 143$; Narum et al., 2013), the other was on cardiac function ($n = 143$; Chen et al., 2018b). All experimental protocols were approved by the University of Idaho's Institutional Animal Care and Use Committee (Protocol #2010-25 and #2013-80).

TABLE 1 Sample size and origin of redband trout *Oncorhynchus mykiss gairdneri* in this study

Analysis		DESERT	Hybrid	MONTCOOL	MONTCOLD	Total
Selection sweep		99	42	96	49	286
Association for thermal tolerance	Thermal tolerance[a]	52	42	49		143
	ACUTE_MORT	16	16	16		48
	ACUTE_SURVIVOR	36	26	33		95
	CHRONIC_MORT	11	3	17		31
	CHRONIC_SURVIVOR	13	7	16		36
Association for cardiac function[c]	Cardiac function[b]	47		47	49	143
	FHMAX_19_LOW	23		23	23	69
	FHMAX_19_HIGH	24		24	25	73
	PEAK_FHMAX_LOW	23		23	23	69
	PEAK_FHMAX_HIGH	24		24	26	74
	T_PEAK_LOW	21		20	23	64
	T_PEAK_HIGH	26		27	26	79

Note:: Three natural redband trout populations from Idaho, USA and a first-generation hybrid strain were used for this study. They are Little Jacks Creek (DESERT), Keithley Creek (MONTCOOL), Fawn Creek (MONTCOLD) and a DESERT × MONTCOOL hybrid strain. The 286 samples were from two previous studies.

[a]One of which measured acute and chronic thermal tolerance.

[b]One measured cardiac function.

[c]Three indices were generated from each fish to evaluate their cardiac performance. Each induce was then grouped into low and high group for association analysis.

2.2 | Phenotype data and groups

Acute and chronic thermal tolerance was previously designed to study transcriptomic responses to thermal stress in DESERT ($n = 52$), MONTCOOL ($n = 49$) and F1_DEST × MTCOOL ($n = 42$). Results have been reported in several publications (Garvin et al., 2015; Narum & Campbell, 2015; Narum et al., 2013). Here, we used the mortality data from acute and chronic thermal exposure in Narum et al., (2013) as phenotypic traits and conducted whole genome resequencing to test for association with survival under thermal stress. Briefly, fry from DESERT and MONTCOOL were collected using electrofisher unit (Model 12-B; Smith-Root, Washington, USA) set at 300 volts and 50 Hz from three streams in Idaho, USA, and were reared at Hagerman Fish Culture Experimentation Station (Hagerman, Idaho, USA) in flow-through spring water at constant 15°C. Gametes were also collected from mature adults in the field and transported to the laboratory for the cross-mate of F1_DEST × MTCOOL. Juvenile fish (fry) were grown to an average weight of 2 g in a common garden setting at 15°C before treatments of heat stress. Daily temperature cycled between 17.5°C (evening) to 28.5°C (afternoon) to mimic the 24 hr water temperature fluctuation in natural desert streams during the hottest summer days (Figure 1b). Fish mortality was checked two times daily and mortality was noted with tissues collected from those fish. Experiments were continued for eight weeks. In the heat stress experiment, two major mortality events were observed (Figure 1b): one occurred within the first week (referred to as "acute" event); the second occurred around one month after experiment between day 27 and 30 ("chronic" event). In the first acute mortality event, individuals were grouped into ACUTE_MORT ($n = 48$ across populations) and ACUTE_SURVIVOR ($n = 95$ across populations) group. In the second chronic mortality event, individuals were grouped into CHRONIC_MORT ($n = 31$ across populations) and CHRONIC_SURVIVOR ($n = 36$ across populations) group (Table 1). By these categorical definitions, some individuals were present in both acute and chronic survival groups.

Cardiac performance data were collected from DESERT ($n = 47$), MONTCOOL ($n = 47$) and MONTCOLD ($n = 49$) redband trout as described in a previous study (Chen et al., 2018b; Figure 1c and d). Briefly, fish were sampled from their native habitats and allowed to rest for a minimum of two hours prior to being anaesthetized in MS-222 solution (65 mg/L). Fish were then moved to a custom-built electrocardiogram (ECG) measuring system, where its heart rate were pharmacologically stimulated to maximum (maximum heart rate or $f_{H,max}$) by atropine sulphate (5.4 mg/kg, Sigma-Aldrich) and isoproterenol (18 μg/kg, Sigma-Aldrich). Water in the ECG system was heated at a rate of 1°C per 6 min until $f_{H,max}$ became arrhythmic, which occurred between 19°C and 28°C. In the course of temperature increase, three indices were used to evaluate cardiac performance for each fish. For the current study, $f_{h,max}$ at 19°C (FHMAX_19) was chosen as a trait that represents cardiac performance at the early warming stage for each individual; peak $f_{h,max}$ (PEAK_FHMAX) is the maximum $f_{h,max}$ of individuals as they reached arrhythmia; the temperature of PEAK_FHMAX (T_PEAK). Within populations, each cardiac phenotype was ranked and then samples were assigned to either a high group (value > median) or a low group (value ≤ median). Sample sizes for each group within and across populations are in Table 1.

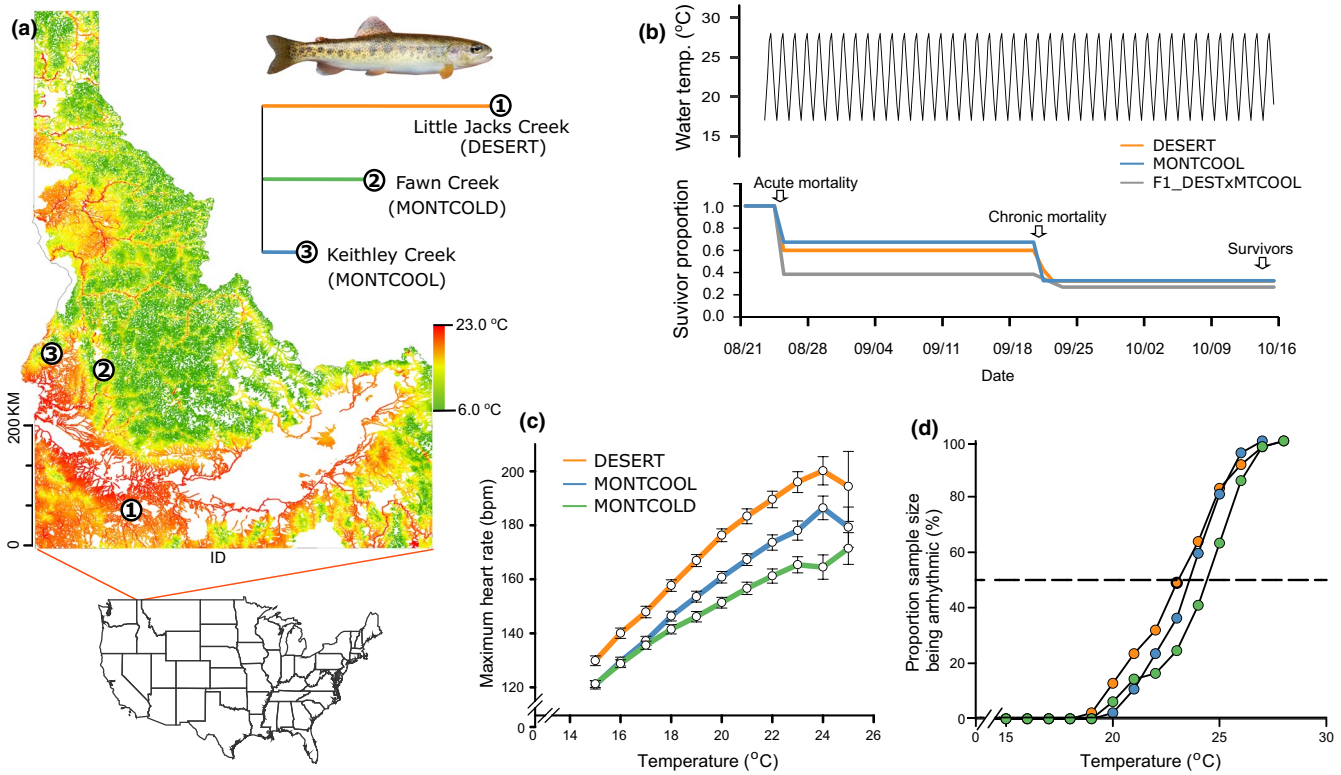

FIGURE 1 Ecological and phenotypic differentiation among redband trout *Oncorhynchus mykiss gairdneri* populations. (a) Redband trout were sampled from ecologically divergent populations in Idaho, USA for population genomics and phenotypic trait association analyses following previous studies (Chen et al., 2018a; Narum et al., 2013). The heat map represents modelled 10 year average of August mean stream temperature for 2002–2011 (Isaak et al., 2016). (b) thermal stress experiment by exposing fish to diel temperature cycles (17°C–28°C). (c) response of pharmacologically-stimulated maximum heart rate ($f_{H,max}$) to warming at the rate of 0.17°C/min. The $f_{H,max}$ is mass corrected to 1 g body mass using exponent of −0.1 (mean ± *SEM*) (d) proportion of fish showing cardiac arrhythmia during warming [Colour figure can be viewed at wileyonlinelibrary.com]

2.3 | Whole genome resequencing and SNP detection

Genomic DNA was extracted from 286 fish using a Chelex extraction method (Sweet et al., 1996). Libraries were prepared with NEBNext Ultra kits with a modified version of the protocol to reduce reaction volumes (see detailed protocol in Horn et al., 2020). Briefly here, DNA of each sample was first quantified and normalized, before being randomly fragmented using dsDNA fragmentase (NEB, Ipswich, Massachusetts). After fragmentation, sequences were end repaired, adapter ligated, and size selected for a mean length of 500 bp. The 286 barcoded samples were sequenced individually with paired-end (2 × 150 bp) reads on eight lanes with the Illumina NextSeq 550 platform with high-output mode. Samples from the same populations were spread across lanes to avoid bias from batch sequencing. After sequencing, reads were demultiplexed by individual barcode.

We used the "PPalign" module in the PoolParty pipeline to perform bioinformatics (Micheletti & Narum, 2018). Briefly, reads were first trimmed by BBduk to remove adapters, contaminants, and low-quality reads (Bushnell, 2016). Reads with average base quality below 20 were filtered. After trimming, reads with length below 25 bp were also removed. Trimmed reads were quality checked by FastQC (Babraham Bioinformatics) and then aligned to *O. mykiss* assembly (GenBank assembly accession: GCF_002163495.1; Pearse et al., 2019) with a minimum mapping quality of 20 using the BWA-MEM algorithm (Li & Durbin, 2009). Duplicate reads were removed by SAMBLASTER (Faust & Hall, 2014) and reads with mapping quality less than five were filtered in SAMtools before being converted to bam files (Li, 2011). Aligned bam files were then sorted in Picard Tools (Broad Institute). Unpaired and unmapped reads were removed in SAMtools. Until this point, sequencing reads were evaluated separately for each sample. These aligned and filtered reads can now be combined for genome scan for population differentiation and trait associations.

2.4 | Genome scan for population differentiation

To prepare for the selective sweep analysis among populations (Analysis I, Figure 2), aligned and filtered reads of samples from the same natural population (DESERT, MONTCOOL and MONTCOLD) were combined. SNPs were called in BCFtools with a SNP quality threshold of 20, and were filtered with read depth threshold to a minimum of 15× to remove low quality reads and a maximum depth of 200× to remove potential paralogous regions (Li, 2011). SNPs

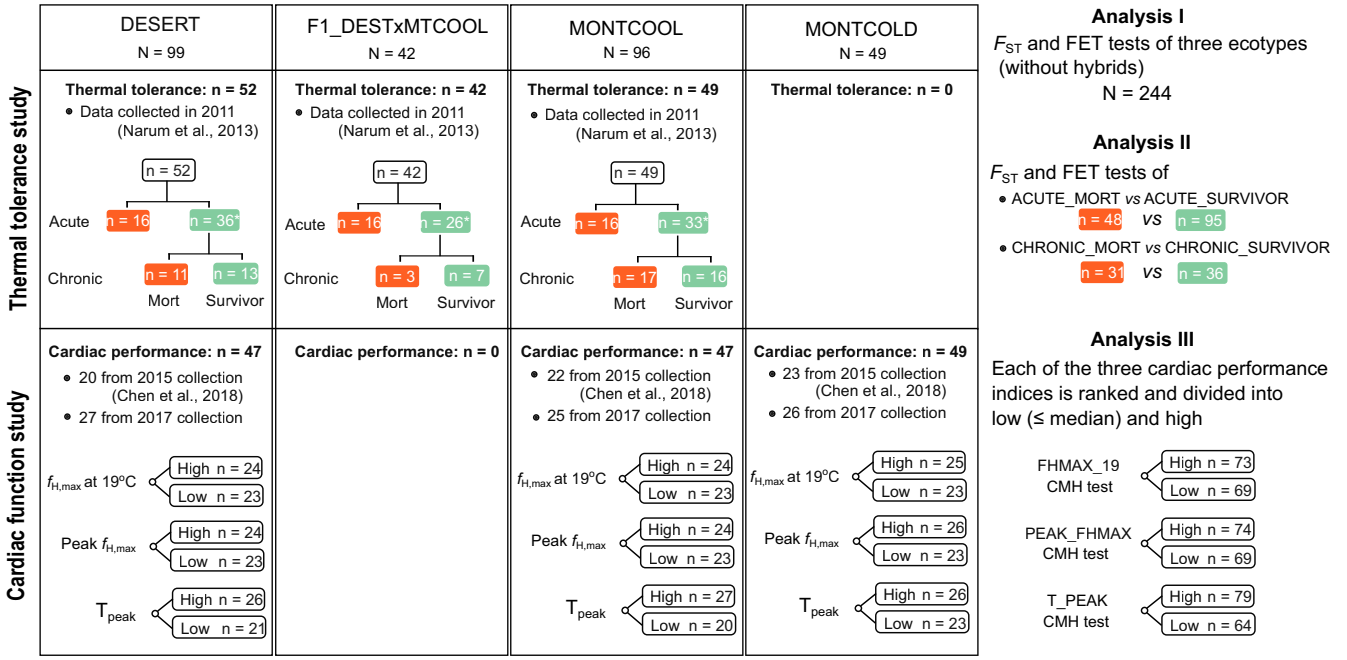

FIGURE 2 Detailed information of redband trout samples in this study and groups used for different analyses included selective sweeps across populations (Analysis I), association tests for survival under acute or chronic thermal stress (Analysis II), and association tests for cardiac phenotypes (Analysis III). There were four groups of redband trout (DESERT, F1_DEST × MTCOOL, MONTCOOL and MONTCOLD) from two studies (thermal tolerance and cardiac function). In each study, multiple phenotypes were measured for association analyses using F_{ST} and Fisher's exact tests [Colour figure can be viewed at wileyonlinelibrary.com]

within the 15 bp window on either side of an indel were also filtered. The population level allele frequencies were then estimated from read counts following procedures in the PoolParty pipeline (Micheletti & Narum, 2018).

Selective sweeps were tested using sliding window Karlsson type F_{ST} (Fixation index) and the significance level was determined using the local score approach (Fariello et al., 2017; Kofler et al., 2011). All analyses genomic data were completed in the PPanalyze module of the PoolParty pipeline (Micheletti & Narum, 2018). The sliding window F_{ST} was calculated from differences in allele frequencies at the window size of 5 kb and step size of 500 bp with methods implemented following PoPoolation2 (Kofler et al., 2011). We used a local score approach to account for linkage disequilibrium in detecting signals of differentiation (Fariello et al., 2017). Local score is the maximum of partial sum of the scores at adjacent loci and determined through the Lindley process over that range. The score of each locus was calculated by a score function of −log10(p-value)-ξ, where p-value is calculated from Fisher's exact test (FET) for each locus. The tuning parameter ξ was included to ensure more than one Lindley process at each chromosome. Because the p-values were different between analyses, ξ values were also different, ranging from 0.6–19. We started each analysis by using a low ξ value, which allows to cumulating large number SNPs and emphasis recent selection events, followed by a higher value, which reduces possible false positive regions and can include possible historic selection events. The most significant regions from tests with different tuning parameters were consistent. The window size of local score approach varies and depends on the cumulative significance level and marker density, and p-values of local scores were computed assuming a Gumbel distribution. For FET p-values that were not uniformly distributed, approximate null distribution of the local scores were based on a resampling procedure using scripts provided by Fariello et al. (2017). The significance threshold of local scores was computed for each chromosome and controlled with a Bonferroni correction ($\alpha = 0.05$).

2.5 | Genome scan for association with acute and chronic thermal tolerance

In genome scan for association with phenotypes, most analyses in this study were specifically between phenotypic groups within the same population to account for population structure among ecotypes. In cases where sample sizes required analyses across populations, signals of significance were evaluated for consistency against analyses without population structure.

Acute and chronic thermal tolerance was tested in DESERT ($n = 52$), F1_DEST × MTCOOL ($n = 42$) and MONTCOOL ($n = 49$) (Analysis II in Figure 2). The genome scan for association was first conducted within each population and then with all population combined. Aligned and filtered reads were combined into ACUTE_SURVIVOR, ACUTE_MORT, CHRONIC_SURVIVOR and CHRONIC_MORT groups. SNPs and allele frequencies were produced using the same approach as that in the population differentiation analysis. We also used the same sliding window F_{ST} and local score approach to scan for genomic regions with significant differentiation in (a) ACUTE_SURVIVOR versus ACUTE_MORT,

and in (ii) CHRONIC_SURVIVOR versus CHRONIC_MORT (sample sizes for each group within and across populations are also in Table 1). The (b) CHRONIC_SURVIVOR versus CHRONIC_MORT analysis was only conduced with all population combined, not within each population due to the small sample sizes in some groups (<15) and low genome proportion coverage at depth of 15×.

2.6 | Genome scan for association with cardiac phenotypes

Cardiac phenotypes were measured in DESERT (n = 47), MONTCOOL (n = 47) and MONTCOOL (n = 49) (Analysis III in Figure 2). The genome scan for association was conducted within each population. Aligned and filtered reads of samples from the same phenotypic group (i.e., either low or high) were combined for each phenotype (i.e., FHMAX_19, PEAK_FHMAX and T_PEAK). SNPs and allele frequencies were produced using the same approach as that in the population differentiation and thermal tolerance association analysis. We also used the same sliding window F_{ST} and local score approach to scan for genomic regions with significant differentiation in high and low group for FHMAX_19 (high FHMAX_19 versus low FHMAX_19), PEAK_FHMAX (high PEAK_FHMAX versus low PEAK_FHMAX), and T_PEAK (high T_PEAK versus low T_PEAK) (sample sizes for each group within and across populations are also in Table 1). We did not perform analysis with all population combined as that in thermal tolerance analysis because sample size is not a limiting factor. Instead, we performed Cochran–Mantel–Haenszel (CMH) approach to test for consistent differences of phenotypic classes across populations (Landis et al., 1978; Mantel, 1963). Specifically, CMH statistic was computed on 3 (populations) × 2 (phenotypic classes, i.e., low and high) × 2 (read count for each of the two alleles) contingency tables.

2.7 | Interpretation of significant genomic signals

To obtain a better understanding of the genetic diversity in significant regions and infer potential evolution and demographic events, we calculated Tajima'D and Nucleotide diversity (pi) at each locus within the peak region for three populations using methods implemented in VCFtools (Danecek et al., 2011). The moving average of mean (10 k window and 5 k step) was calculated using a Gaussian kernel smoothing function. A neighbour joining tree was built based on Nei's genetic distance and 10,000 bootstraps from putative adaptive loci from each significant region using the PPanalyze module from the PoolParty pipeline (Micheletti & Narum, 2018). Candidate genes that were identified within 60 kb range of peak signals were then annotated based on existing information for the reference genome assembly of *O. mykiss* available from NCBI (GCF_002163495.1; Pearse et al., 2019).

2.8 | Candidate marker development

To enable future studies to further investigate the significant association of *cerk* gene with thermal phenotypes, eight candidate markers from the region on chromosome 4 were developed and genotyped for all individuals in the study (n = 286). Genotyping was conducted using the genotyping-in-thousands by sequencing (GT-seq) protocol (Campbell et al., 2015). Briefly, primers were designed to amplify the most significant SNPs from chromosome 4 in genome scan analyses. Primers were then added to an existing GT-seq panel (Collins et al., 2020) to efficiently genotype individual *O. mykiss* samples. Amplicons were prepped following standard GT-seq protocols and sequencing was conducted on an Illumina NextSeq 550 platform. Reads were quality filtered and genotypes were called for each individual with the GT-seq pipeline (Campbell et al., 2015). Given that large sample sizes are needed for individual association analyses (e.g., Ball, 2013; Josephs et al., 2017), the markers developed here will be used in future studies but were not tested for association with the relatively small samples sizes for phenotypic groups in the current study.

3 | RESULTS

3.1 | Whole genome resequencing and SNP detection

The 286 fin clip samples were sequenced in eight sequencing runs, which generated reads ranging from 450 M to 600 M reads per run. The average reads per individual was 11 M. Six individuals had less than 1 M reads. Others had read numbers ranging between 1 M–35 M. After combining normalized reads of individuals from the same population, each population had 573.3 M–835.8 M reads, providing ~75% genome coverage at a depth of 15× (Table S1).

3.2 | Genome scan for population differentiation

Genome scans for significant differentiation among populations (selective sweeps; Analysis I, Figure 2) pointed to six regions on chromosome 3, 4, 14, 23, 26 and 28 (Figure 3). There was a total of six candidate genes based on peak local score across populations (Table S2) while pairwise comparisons of populations demonstrated a few consistent significant regions (Figure S1). The most significant signal was on chromosome 28 that contains genes of *greb1l* (growth regulating estrogen receptor binding 1) and *rock1* (Rho-associated protein kinase 1). The second most significant region was on chromosome 4 (8.1 M–8.6 M) that encodes the *cerk* gene (ceramide kinase). Other significant signals on chromosome 3, 14, 23 and 26 did not include underlying gene coding sequences and were relatively distant from annotated genes (>60 kb).

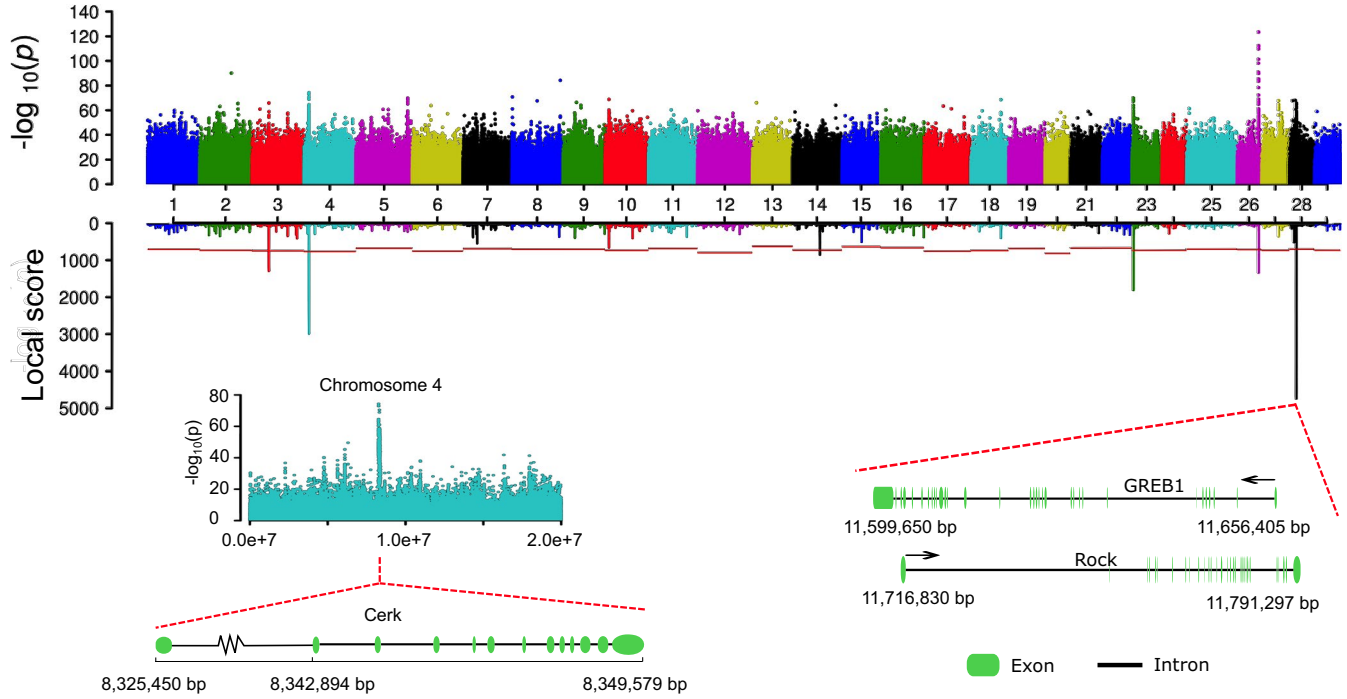

FIGURE 3 Manhattan plots for differentiation among redband trout populations (Analysis I). The upper portion (a) illustrates *p*-values of individual SNPs from Fisher's exact test and the lower part (b) is the local maximum score based on multiple markers in physical linkage (based on of Lindley process with $-\log_{10}(p)$-19.0 function). Red lines in lower Manhattan plot are thresholds of local scores computed for each chromosome [Colour figure can be viewed at wileyonlinelibrary.com]

3.3 | Genome scan for association with acute and chronic thermal tolerance

Association tests for combined acute mortality and survivor groups (Analysis II, Figure 2) had reads of 286 M and 620 M, providing 60% and 73% genome coverage, respectively, at depth of 15× or greater (Table S3). Combined chronic mortality and survivor groups had reads of 220 M–243 M, providing 48% and 53% genome coverage at depth of 15× or greater. Results from both analyses identified the same genomic region on chromosome 4 (8.1 M–8.6 M; Figure 4) as highly significant (especially within the DESERT population; Figure S2), which is the same *cerk* gene region as that in the initial population divergence analysis. Other candidate genes based on peak local score were detected but at much lower significance level (Tables S4 and S5).

3.4 | Genome scan for association with cardiac phenotypes

Association tests for cardiac phenotype groups (Analysis III, Figure 2) had reads ranging from 202 M to 324 M, providing 43% and 66% genome coverage at depth of 15X or greater (Table S6). In response to increasing temperatures in the experiment, an expected pattern of cardiac function was observed where heart rate $f_{h,max}$ increased with warming temperatures for all fish (Figure 1c, d and Figure S3). Fish start showing arrhythmia when temperatures reached 19°C, but arrhythmia occurred for individuals at temperatures that ranged between 19°C–28°C. Groups of cardiac phenotypes within each population had genome coverage ranging from 43% to 76% at depth of 15X or greater. In the association analysis for three phenotypes, CMH tests highlighted that FHMAX_19 and PEAK_FHMAX had consistent association with *cerk* gene across populations (Figure 5). Beside *cerk*, there were also population specific strong signals that were associated with cardiac phenotypes. That included regions on chromosome 12 for MONTCOOL (Figure S4), chromosome 25 for PEAK_FHMAX (Figure S5) and chromosome 3, 25, 16 for T_PEAK (Figure S6). Annotations of significant regions within each population were summarized in Tables S7-S9 (FHMAX_19), Tables S10-S12 (PEAK_FHMAX) and Tables S13-S14 (T_PEAK).

3.5 | Genetic diversity at *cerk* gene region

At the *cerk* gene region, Tajima'*D* was positive in the desert population, but negative in the montane populations, suggesting evidence for balancing selection (Figure 6a). The desert population also had an increased nucleotide diversity relative to the other montane populations (Figure 6b). Neighbour joining trees constructed from markers from the significant *cerk* region clustered the same phenotypic classes from different populations together (Figure 6c).

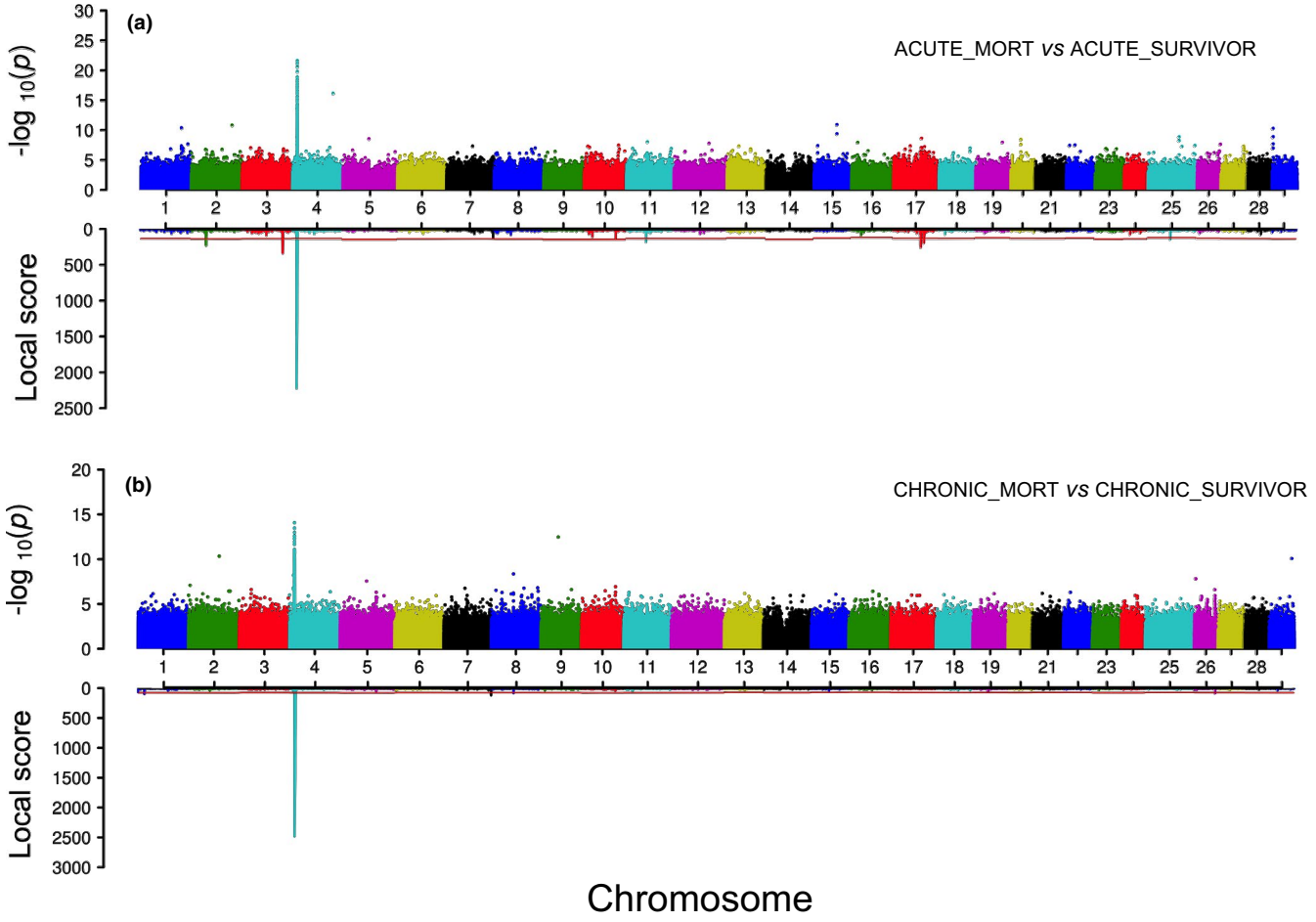

FIGURE 4 Manhattan plots illustrating genetic difference between redband trout mortalities (morts) and survivors after (a) acute and (b) chronic exposure to thermal stress up to 28°C (Analysis II). In each panel, the upper plots are the *p*-values from Fisher's exact test and the lower ones are the local maximum score (based on Lindley processes with $-\log_{10}(p)$-0.7 (acute analysis) and $-\log_{10}(p)$-0.8 (chronic analysis). Red lines in lower Manhattan plot are thresholds of local scores computed for each chromosome [Colour figure can be viewed at wileyonlinelibrary.com]

3.6 | Candidate marker development

The eight candidate markers from *cerk* gene were genotyped successfully in nearly all samples. Of the 286 samples in this study, genotyping success rate (GSR) ranged between 78.0%–81.5% for the eight candidate markers (Table S15). All markers had similar minor allele frequencies (0.06–0.07).

4 | DISCUSSION

In this study we used lcWGR on individual fish to estimate allele frequencies for high density genome markers and discovered putative genes that may be involved in thermal adaptation in redband trout populations from hot desert, cool montane and cold montane climates. In addition to scans for genomic differentiation between populations, this study included association tests with phenotypic traits for thermal adaptation that were characterized in previous studies (Chen et al., 2018a,2018b; Narum & Campbell, 2015; Narum et al., 2013). This approach revealed a consistent candidate gene (*cerk*) on chromosome 4, which was not only among the most differentiated region between populations, buy also significantly associated with acute and chronic thermal tolerance, as well as cardiac performance under thermal stress.

Previous studies have examined thermal adaptation in aquatic ectotherms and suggested a polygenic basis for this trait (Chen et al., 2018a; Jackson et al., 1998; Narum et al., 2013). Most genetic analyses in redband trout have produced a large number of significant signals across the genome with limited precision (Chen et al., 2018a; Narum et al., 2013). One advantage of high-density genomic markers is being able to identify candidate regions from multiple significant variants within linkage blocks as opposed to reduced representation sequencing that provides fewer SNPs in the genome. Significant signals from this study were not identified in a previous study on the same populations (Chen et al., 2018b), which used RAD-seq to sequence 100 bp segments around *Pst*-I restriction enzyme recognition sites dispersed throughout the genome and included multiple SNPs per tag and a total of 526,301 SNPs. We estimate that the Chen et al. (2018b) study sequenced less than 3% of the total genome based on the predicted frequency of *Pst*-I

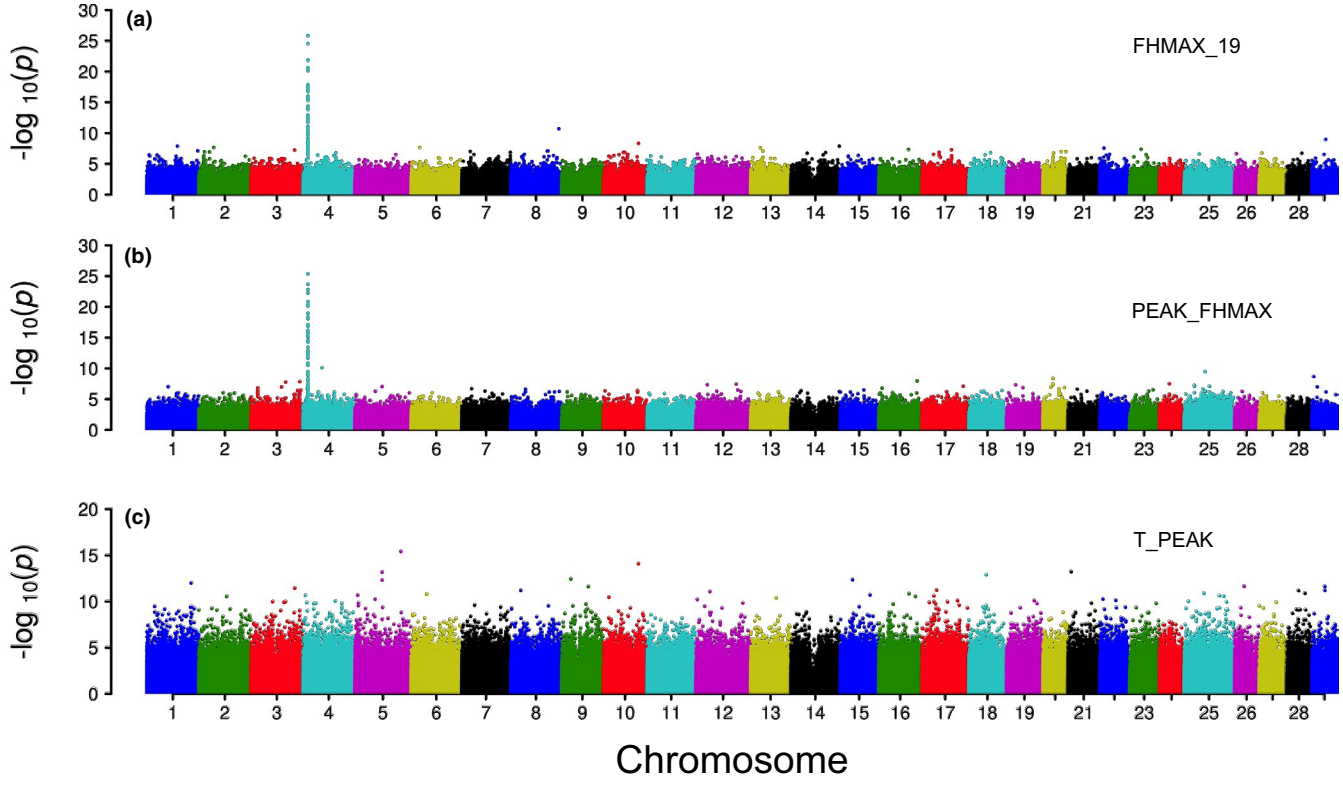

FIGURE 5 Manhattan plots for Cochran-Mantel-Haenszel (CMH) tests of genetic differences between redband trout grouped by phenotypic classes of cardiac performance within each population (Analysis III). Three cardiac performance traits are: (a) maximum heart rate at 19°C (FHMAX_19); (b) peak $f_{H,max}$ (PEAK_FHMAX); (c) temperature of peak $f_{H,max}$ (T_PEAK) [Colour figure can be viewed at wileyonlinelibrary.com]

cut sites in a 2.4 Gb genome for *O. mykiss*. Specifically, the 500 kb region on chromosome 4 at the candidate *cerk* gene had 2,870 SNPs in the current study while this same region only had 123 SNPs from Chen et al. (2018b) (Figure S7). Thus, while the marker density in Chen et al. (2018b) appears high, the actual portion of the genome was relatively low and few markers would be expected per linkage block. In the current study, the resequencing approach covered approximately 43%–73% of the genome with more markers per linkage block, which allowed for improved statistical power to detect signals of selection using the local score approach (Fariello et al., 2017).

In addition to divergence in allele frequencies observed at chromosome 4, we found that genetic diversity contributing to the divergence in this genomic region is higher in fish from the hot desert climate (Little Jacks Creek). This is probably a result of balancing selection rather than directional selection according to the Tajima'D results. Mechanistically, redband trout are ectotherms living in shallow streams, where fish are frequently exposed to environmental extremes in both summer and winter seasons. Therefore, a population of specialists for hot summer conditions may be less favored by the desert environment compared to generalists that are adapted to high variation in stream temperatures in both cold and hot extremes (Angilletta et al., 2003).

Identification of the candidate region on chromosome 4 provides an example of population divergence with potential fitness-related traits. A previous study using whole genome pool-seq also identified significant divergence in *cerk* gene between ancestral California steelhead *O. mykiss* and introduced steelhead in Lake Michigan (Willoughby et al., 2018), which implied the role of *cerk* in the process of adaptation to new environmental stressors. However, since the Willoughby et al. (2018) study included neither phenotypic data or compared introduced populations to source populations, it is uncertain what mechanism or phenotype was driven by the adaptive signal that was detected. Nevertheless, Willoughby et al. (2018) describe gene functions of *cerk* including metabolic activity that could be envisioned to be involved with thermal adaptation as shown in the current study.

Function of the *cerk* gene in thermal adaptation is still yet to be explored, especially in non-model organisms. The protein product of *cerk* gene is ceramide kinase, which catalyzes ceramide to ceramide-1-phosphate. Ceramide is a member of sphingolipids, which is a class of important signaling lipids that regulate a wide spectrum of cellular activities (Chalfant & Spiegel, 2005), including stress-related signaling transduction pathways (Hannun, 1996; Ruvolo, 2001). The most relevant function of Cerk to the present study is its protective role on cardiovascular systems (Cannavo et al., 2017; Knapp, 2011). Since heart is the last organ receiving oxygen in fish circulatory systems and oxygen becomes limited at high temperatures, the divergence in redband trout population and cardiac function might be related to the intracellular regulation of ceramide and ceramide-1-phosphate by Cerk. Other described putative functions

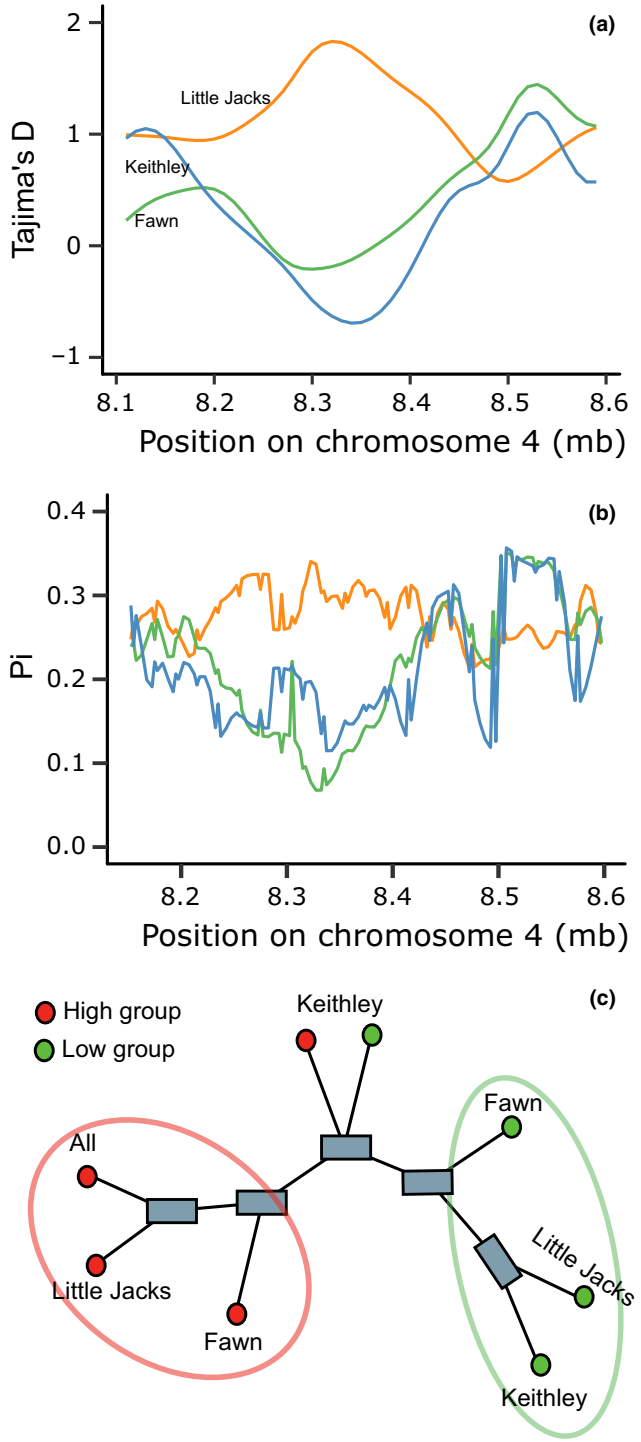

FIGURE 6 Genetic diversity analyses of redband trout populations in a region of chromosome 4:8.1 M to 8.4 M. (a) Tajima'*D*. (b) nucleotide diversity (pi). Each line is the moving average of mean (10 k window and 5 k step) for one population. (c) Neighbour joining tree is built from putative adaptive loci from this region. The NJ tree is similar when analysed for thermal tolerance and cardiac function, with phenotypes classified as either high (red) or low (green) [Colour figure can be viewed at wileyonlinelibrary.com]

of ceramide-1-phosphate is a ligand for G-protein-coupled receptors and thus involved in an array of important cellular functions (Spiegel & Milstien, 2002). In terms of regulation in cell survival and growth, ceramide tends to inhibit cell growth and efficiently induces apoptosis; while ceramide-1-phosphate stimulates cell proliferation (Gómez-Muñoz, 2006). Therefore, the *cerk* gene acts as a rheostat of cellular homeostasis. There are also accumulating studies on the role of ceramide in heat shock response, which have been summarized in several reviews (Jenkins, 2003; Nikolova-Karakashian & Rozenova, 2010). For example, in human cells, ceramide reduces the heat shock response, mainly by suppressing the expression of *heat shock protein 70* (*hsp70*) (Kondo et al., 2000), which has a protective effect on protein processing under heat stress conditions. In rat liver, ceramide produces oxidative stress by directly influencing electron transport chain in mitochondria and causing the accumulation of reaction oxygen species (ROS) (García-Ruiz et al., 1997). Taken together, evidence in mammalian literature and findings from the present study suggest that *cerk* gene could play a role in responses to environmental stressors including temperature tolerance in redband trout and possibly other ectotherms. Despite the strong signal detected for *cerk* on chromosome 4, it is possible that neighbouring genes in strong LD could be involved with thermal adaptation, or it is possible that gene duplication or structural variation in this region could have caused unbalanced allele frequencies. Thus, further verification of association and gene function for *cerk* is needed to provide insights to its role in cardiac performance and thermal tolerance. The candidate markers we developed for GT-seq provide an opportunity to further investigate the association of *cerk* with large samples sizes since they can be genotyped cost effectively in thousands of samples with amplicon sequencing approaches (Campbell et al., 2015; Meek & Larson, 2019).

Another significant region on chromosome 28 points to the *greb1* and *rock1* gene, which has been previously shown to be associated with migration timing in anadromous steelhead (Hess et al., 2016; Micheletti et al., 2018; Prince et al., 2017) and chinook salmon (Narum et al., 2018). It is therefore not surprising that we did not find significant associations between *greb1/rock1* gene and phenotypic traits related to thermal tolerance. Instead, the significant signal at this region on chromosome 28 indicates that redband trout populations harbour variation for early and late adult migratory phenotypes but it is unknown how this trait might be expressed in these resident fish. Since habitats of redband trout populations included in this study were historically accessible to the anadromous form before human-caused barriers were created (e.g., Hells Canyon Dam), it is possible that the differences observed at *greb1/rock1* gene were related to migration timing in the historic anadromous era rather than a result of contemporary thermal adaptation. However, different spawning time has been observed among redband trout populations in Montane, USA, which largely depends on when local water temperatures reach a mean of approximately 6.0°C–8.2°C and occurs earlier for lower elevation streams (Muhlfeld, 2002). In spite of the association with life history characteristics, the role of *greb1/rock1* gene in thermal adaptation warrants more study.

The genetic architecture of thermal adaptation is still largely unknown in most ectothermic species. Along with previous studies on

this subject (Chen et al., 2018a), this study identified multiple significantly differentiated genomic regions among populations which suggest a polygenic basis of thermal adaptation in redband trout populations. Three of the regions did not include coding genes, suggesting they might be regulatory elements of adjacent genes. For example, gene expression pattern (e.g., heat shock protein genes and beta adrenergic pathway genes) in response to temperature has been found to be different among populations (Chen et al., 2018a; Narum & Campbell, 2015). However, no genes were found to be previously identified genes that were differentially expressed. It is also possible that some of these regions were false positives due to demographic history (e.g., population bottlenecks), especially when redband trout effective population size is not large in most streams. However, peaks on chromosome 4 and 28 were colocalized with protein coding genes and demonstrate consistent evidence for association analyses, making them strong candidates associated with phenotypic variation. Nevertheless, our results pinpointed candidate genes and we generated primers for markers which can be used to for survey more populations across a broader ecological range for conservations purposes. It can also be used as a genomic tool to screen source populations for genetic rescue or artificially assisted reintroduction or migration activities (Allendorf et al., 2010).

The design of our study was intended to overcome challenges in detecting candidate genes involved in local adaptation that are often constrained by low power due to modest marker density in species with extent of linkage disequilibrium that is low, or individual association tests for small sample sizes with known phenotypes (Hoban et al., 2016; Lowry et al., 2017; Tiffin & Ross-Ibarra, 2014). While we achieved high marker density and utilized a statistical testing framework based on differences in allele frequencies among groups rather than individual genotypes, there were caveats to our approach. Caveats include inability to estimate genome levels of LD or effect size of the *cerk* gene based on allele frequencies, and lack of complete independence among samples used for selective sweep and association tests. However, we developed candidate markers that will enable future studies to address some of these caveats by genotyping large numbers of individuals with known phenotypes for independent analyses and estimating LD. Improvements to sequencing may also enable whole genome sequencing at high enough read depth to allow genotyping of millions of SNPs at the individual level. One of the most challenging aspects of future studies of thermal adaptation is expected to be collection of phenotypic data for large numbers of individuals since this is an extremely time-consuming component. Thus, phenotyping large sample sizes is expected to be a continued constraint but advances in technology such as passive integrated transponder tags that wirelessly monitor heart rates are anticipated to improve collection of individual phenotypic data.

In conclusion, results from this study identified a candidate genetic mechanism of thermal adaptation at a specific genomic region on *O. mykiss* chromosome 4 that encodes the *cerk* gene. Notably, this gene is significantly associated with fitness related traits (cardiac function and thermal tolerance); it is also significantly differentiated between conspecific redband trout populations that are locally adapted to different stream temperatures. However, the function of *cerk* remains unknown in this species and further validation is needed. Our allele frequency based results can be verified by individual genotype to phenotype association tests with *cerk* markers that can be genotyped more cost effectively with methods such as amplicon sequencing (Campbell et al., 2015). Validated markers are expected to be effective to examine questions regarding adaptive capacity of non-model species under scenarios of climate change (e.g., Bay et al., 2017). More broadly, with the advancement of whole genome resequencing and bioinformatics we anticipate that genetic architecture of complex traits will gradually be discovered that can be utilized for conservation applications (Waples et al., 2020).

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AUTHOR CONTRIBUTIONS

Z.C. performed data analyses and wrote the manuscript. S.R.N. provided funding and assisted Z.C. in experimental design and manuscript preparation. Both authors have read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

Sequence data are available from NCBI SRA database with project accession number of PRJNA600878 (Chen, 2020b). SNPs and corresponding allele frequency data for all have been submitted to Dryad, https://doi.org/10.5061/dryad.bk3j9kd73 (Chen, 2020a). Phenotypic data was also deposited to Dryad, https://doi.org/10.5061/dryad.xwdbrv1c7 (Chen, 2020c).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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