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Adaptation of redband trout in desert and montane environments

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Abstract

Natural populations that evolve under extreme climates are likely to diverge because of selection in local environments. To explore whether local adaptation has occurred in redband trout (*Oncorhynchus mykiss gairdneri*) occupying differing climate regimes, we used a limited genome scan approach to test for candidate markers under selection in populations occurring in desert and montane streams. An environmental approach to identifying outlier loci, spatial analysis method and linear regression of minor allele frequency with environmental variables revealed six candidate markers ($P < 0.01$). Putatively neutral markers identified high genetic differentiation among desert populations relative to montane sites, likely due to intermittent flows in desert streams. Additionally, populations exhibited a highly significant pattern of isolation by temperature ($P < 0.0001$) and those adapted to the same environment had similar allele frequencies across candidate markers, indicating selection for differing climates. These results imply that many genes are involved in the adaptation of redband trout to differing environments, and selection acts to reinforce localization. The potential to predict genetic adaptability of individuals and populations to changing environmental conditions may have profound implications for species that face extensive anthropogenic disturbances.

Keywords: genome scan, local adaptation, *Oncorhynchus mykiss*, thermal tolerance

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Introduction

In nature, widely distributed species often occupy environments outside their optimal preference, causing selective pressure for physiological and genetic adaptation. This is especially true for species that are heavily impacted by human disturbances that may expose them to more extreme environments than normally observed, and adaptation to differing habitat may be further reinforced by limited gene flow because of habitat fragmentation, dispersal barriers, or relocation to new areas. Under these circumstances, selective pressure has typically been shown to be divergent (e.g., Hendry *et al.* 2008) but balancing selection may also play a role to maintain genetic variation (e.g., MHC genes and immune response; Piertney & Oliver 2006). While selection and subsequent genetic adaptation has been well demonstrated under laboratory conditions for model organisms such as *Drosophila* spp. (e.g., Hoffmann *et al.* 2003) and zebrafish (e.g., Neuhauss *et al.* 1999), adaptation in natural populations of non-model species is difficult to study. This is because of several confounding factors such as the inability to isolate variables in complex environments, uncontrolled mating (predetermined parental crosses are not possible), generation length and limited genome information (i.e., annotated sequence, quantitative trait loci and linkage maps).

Genome scans with many genetic markers provide the opportunity to investigate local adaptation in

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natural populations and identify candidate genes under selection. This has become a commonly employed approach in ecological and population genetics studies to detect outlier loci that are putatively under selection (e.g., Vasemagi & Primmer 2005; Nosil *et al.* 2008). A variety of tests have been developed to identify 'general' outlier loci from a neutral distribution (Beaumont & Nichols 1996; Beaumont & Balding 2004; Foll & Gaggiotti 2008; Excoffier *et al.* 2009), and additional tests are also available to identify loci associated with specific environmental variables (e.g., Joost *et al.* 2008). Additionally, correlation methods can be highly informative to identify markers in coding and cis-regulatory regions of known functional genes that are associated with specific selective pressures or phenotypes (Lyman & Mackay 1998; Umina *et al.* 2005; Chase *et al.* 2009; Torgerson *et al.* 2009). With increasing genomic information available for non-model organisms, single-nucleotide polymorphisms (SNPs) have begun to see increased use as genetic markers for population genetic studies (e.g., Luikart *et al.* 2003; Morin *et al.* 2004). These sequence polymorphisms are dense throughout the genome of most organisms and are commonly observed in both coding and non-coding regions of functional genes, making them ideal markers to study adaptive molecular variation (e.g., Akey *et al.* 2002). In a large suite of unlinked SNPs that are distributed across the genome, it is possible to utilize both functionally neutral and adaptive markers within a single study. This combination of information provides a powerful approach to study questions in ecological genetics because both demographic processes (i.e., gene flow and genetic drift) and local adaptation (i.e., selection) may be inferred. In this study, we employed a combination of these approaches to test for neutral and adaptive genetic differences in redband trout (*Oncorhynchus mykiss gairdneri*) inhabiting different thermal environments.

Climate has been shown to influence a variety of traits in fish including thermal tolerance, growth, development and disease resistance (Crozier *et al.* 2008). Cellular response to thermal stress and adaption to extreme temperatures has been shown to be polygenic in fish, with genes involved from many different biological pathways. Under heat stress, a wide variety of genes are differentially expressed including those related to immune response, signal transduction, protein processing, response to stress and metabolism (Kassahn *et al.* 2007). Adaptation to cold stress has also been shown to be necessary for organisms occupying very cold temperatures (Ciardiello *et al.* 2000). Water temperature can affect growth and development rates in fish, with warmer water resulting in accelerated rates unless temperature exceeds optimal levels and causes stress (e.g., Beer & Anderson 2001). Studies have shown that fry emergence timing is associated with water temperature and often matches local conditions through either spawning date or embryo development (e.g., Brannon 1987; Beacham & Murray 1990). Warm or cold water temperatures may also cause higher pathogen population growth rates and increased likelihood of disease in fish (Holt *et al.* 1989; Marcogliese 2001). Resistance to diseases can increase in fish populations following exposure to various pathogens (Zinn *et al.* 1977), thus resulting in adaptation to local pathogens. Therefore, extensive selective pressure can occur for several traits as a result of differing climates throughout a species' range.

Redband trout occupy a wide range of habitats including desert and montane streams, with significant differences in habitat characteristics such as elevation, gradient, substrate, shading and temperature (Meyer *et al.* 2010). Physiological differences have also been observed in redband trout occupying desert streams (Gamperl *et al.* 2002). These studies indicate the potential for local adaptation of redband trout to differing habitats across environmental gradients. In this study, individual redband trout were sampled across several tributaries representing desert and montane streams and screened with 96 SNP markers from functional genes of diverse biological pathways. We tested predictions that variable climates would result in divergent selection and local adaptation of redband trout to each environment.

Methods and materials

Collection sites

A total of 499 individual redband trout were sampled across 12 tributaries from southwest Idaho, USA, representing populations that were preclassified as desert or montane streams (six sites each) as determined by geographic location (Fig. 1). Individual fish were sampled by electro-fishing in multiple reaches within a site to avoid sampling related individuals. Fish were a mix of age classes as determined by length frequency and sampled during the summer seasons of 2002–2005. A non-lethal fin clip was collected from each fish as sample tissue and immediately preserved in 100% non-denatured ethanol.

A total of nine environmental variables were recorded to characterize habitat of each collection site (Table 1). Latitude and longitude coordinates were recorded for each collection area with a field GPS instrument and used to estimate elevation, temperature and precipitation (Table 1). Elevation was determined from a U.S. Geological Survey 10-m digital elevation model. Annual average maximum air temperatures

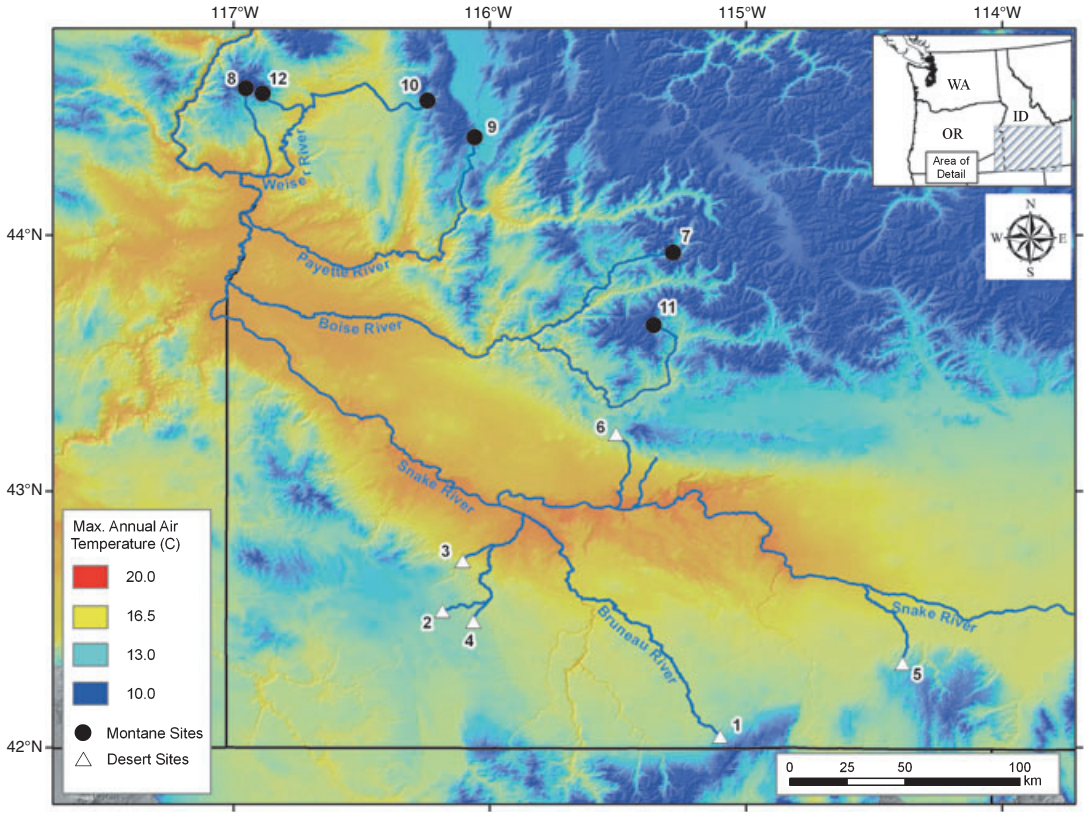

Fig. 1 Map of the study area in southwest Idaho, with inset of Pacific Northwest USA. Sites where redband trout (*Oncorhynchus mykiss*) were collected are numbered and correspond to stream locations in Table 1. Sites classified as desert or montane streams are indicated by either white triangles or black dots, respectively.

(hereafter referred to as 'air temperature') and annual total precipitation were simulated at 800-m cell resolution from a model based on climate normals from a 30 -year period (1971–2000) in PRISM (parameter-elevation regressions on independent slopes model; http://www.prism.oregonstate.edu/) of the Oregon Climate Service. Daily stream temperatures were collected from each site from May to October 2009 to validate the use of daily and long-term air temperatures for analysis. Stream temperature data were unavailable from Fawn Cr. as thermal logger devices at that site were tampered with and destroyed. Additional habitat data was collected for each site as described in Meyer *et al.* (2010) for six variables (stream gradient, specific water conductivity, stream width, per cent fine sediment, per cent gravel and per cent cobble/boulder). Briefly, stream gradient was determined by elevational increments bounding each site by approximately 1 km, specific conductivity (μS/cm) was measured with a conductivity meter (accurate to ±2%), stream width was estimated by averaging 10 transects from each site, and per cent substrate composition was visually estimated within 1 m of transects at each site. All nine environmental variables were tested for differences between desert and montane collections with ANOVA (d.f. = 11).

SNP genotyping and descriptive statistics

Tissue samples from each individual were processed with Qiagen DNeasy® kits to extract DNA from fin clips stored in 100% ethanol. Isolated DNA from each sample was genotyped for 96 SNP markers (see Table S1, Supporting Information) with Taqman chemistry (Applied Biosystems) and Fluidigm 96.96 dynamic array chips (reaction volumes of ~7 nL) for SNP genotyping. As genotyping in nL reaction volumes reduces the average starting copy number to a range where genotyping accuracy becomes less reliable (Campbell & Narum 2009a), a pre-amplification protocol was used to increase the number of starting copies. Pre-amplification occurred in 7 -μL reactions with 2 μL of genomic DNA and 5 μL of PCR cocktail (3.5 μL of Qiagen Multiplex Mastermix, 0.875 μL of 96 pooled primer sets at 0.36 μM, and 0.625 μL water) under the following thermal cycling programme: initial denature at 95 °C for 15 min, 14 cycles of 95 °C for 15 s and 60 °C for 4 min,

Table 1 Sampling locations and environmental variables for redband trout collected in drainages of southwest Idaho, USA

Map no.	Region	Collection	Drainage	Latitude/longitude	*n*	Mean annual max. air temp (°C)	Elevation (m)	Gradient (%)	Specific conductivity (μS/cm)	Width (m)	Fines (%)	Cobble/ gravel (%)	Boulder (%)	Mean annual precipitation (cm)
1	Desert	Deer Cr.	Bruneau R.	42.043633/−115.09903	54	13.8	1842	1.9	71.0	1.3	5.0	25.0	57.0	45.2
2	Desert	Big Jacks Cr.	Bruneau R.	42.532970/−116.18385	30	14.5	1704	3.2	150.0	1.6	5.0	26.0	54.0	38.1
3	Desert	Little Jacks Cr.	Bruneau R.	42.728700/−116.10516	60	16.8	1081	1.4	124.0	2.3	9.0	25.0	31.0	26.9
4	Desert	Duncan Cr.	Bruneau R.	42.492565/−116.06401	73	15.0	1638	1.8	134.0	2.4	25.0	38.0	36.0	31.2
5	Desert	McMullen Cr.	Snake R.	42.329260/−114.38737	28	14.2	1486	0.3	108.0	1.8	7.0	56.0	31.0	32.4
6	Desert	Bennett Cr.	Snake R.	43.224200/−115.50603	30	14.6	1406	1.1	95.0	2.0	24.0	22.0	5.0	54.9
7	Montane	Johnson Cr.	Boise R.	43.936886/−115.28058	59	13.4	1716	2.1	n/a	n/a	n/a	n/a	n/a	82.7
8	Montane	Upper Manns Cr.	Weiser R.	44.574870/−116.95055	31	12.1	1502	1.5	120.0	4.7	0.0	27.0	37.0	87.4
9	Montane	Fawn Cr.	Payette R.	44.382336/−116.05894	30	12.2	1596	0.6	25.0	3.2	17.0	54.0	6.0	73.8
10	Montane	L. Weiser Cr.	Weiser R.	44.525684/−116.24255	29	13.3	1377	3.5	65.0	8.4	3.7	7.9	78.9	69.2
11	Montane	Whiskey Jack Cr.	Boise R.	43.649063/−115.35925	53	13.0	1714	3.7	58.0	4.1	15.0	39.0	33.0	85.5
12	Montane	Keithley Cr.	Weiser R.	44.553380/−116.88535	22	13.0	1370	5.5	96.0	3.4	7.0	27.0	48.0	87.8

n/a, data not available.

hold at 4 °C. Immediately after cycling, 133 μL of nuclease-free H_2O or TE buffer was added to each PCR and stored at 4 °C.

Pre-amplified template DNA was then genotyped with Fluidigm 96.96 dynamic array chips that included a three-step process: (i) SNP assays (Taqman primers/probes) and DNA samples were mixed according to manufacturer's protocols and loaded onto the chip with a Fluidigm IFC Controller instrument, (ii) target SNPs were amplified for 50 cycles on an Eppendorf thermal cycler specially formatted for the Fluidigm 96.96 chip, and (iii) chips were scanned with a Fluidigm EP-1 instrument to detect fluorescently labelled allele-specific probes. Genotypes for each assay were auto-scored with Fluidgim SNP Analysis v.2.1.1 software and verified by eye with scoring guides provided by an assay database and a heterozygous indicator sample for each SNP. As the SNP markers used in this study were ascertained from a broad panel of samples including related populations from the Columbia River (e.g., Campbell *et al.* 2009), ascertainment bias should be limited. Any potential ascertainment bias should effect populations in this study equally because none were directly included in SNP discovery.

Tests for linkage disequilibrium between all pairs of loci were also performed using the MCMC approximation of the exact test in GENEPOP v. 3.3 (Raymond & Rousset 1995). Because multiple comparisons were involved, correction against Type I error was made in both tests with the B-Y FDR method (False Discovery Rate; Benjamini & Yekutieli 2001) that provides increased power relative to the Bonferroni method (Narum 2006).

A total of 96 SNPs were screened in this study, but 20 markers were dropped from further statistical analyses (see Table S1 for list, Supporting Information). Five SNPs were dropped from analyses because of poor amplification and low-quality genotype plots. Three SNPs in the panel were used to detect potential hybrids of *Oncorhynchus mykiss* and *Oncorhynchus clarki* (cutthroat trout) but dropped from further analysis because hybrids were not identified in this study. Eight pairs of SNPs were known to be physically linked on the same gene and only the most informative of each pair was included in the analyses. An additional four pairs of SNPs had highly significant linkage disequilibrium ($P < 0.0001$), and the least informative of each pair was dropped from further analyses. A total of 76 remaining SNPs were included in the subsequent statistical tests (Table S1, Supporting Information).

Deviation from Hardy–Weinberg equilibrium was evaluated at each locus and population with the Markov Chain Monte Carlo (MCMC) approximation of Fisher's exact test implemented in GENEPOP v. 3.3 (1000

batches with 1000 iterations; Raymond & Rousset 1995). Minor allele frequency (MAF), unbiased heterozygosity (H_E) and global F_{ST} were estimated for each SNP in each collection with GENEPOP. Effective population size (N_e) was estimated for each collection with the program LDNE (Waples & Do 2008). To determine confidence interval (CI) for N_e, we used the option of 'jackknife CIs' that corrects for bias in parametric CIs when LDNE estimates are based on many loci (Waples 2006). We present results from setting the minimum frequency of alleles included in the analysis (P_{crit}) to a value of 0.02, which is a middle-range default value of the program. The P_{crit} value chosen was a compromise between a higher value that will lower the upward bias of N_e and a lower value that increases the precision of the estimate (Waples & Do 2008). Differences in H_E and N_e were tested with ANOVA (d.f. = 11).

We investigated patterns of deviation from neutral expectations among the 76 SNPs with an outlier approach based on simulation methods initially proposed by Beaumont & Nichols (1996). This approach was implemented in LOSITAN (Antao *et al.* 2008) that simulates a distribution of F_{ST} values under neutral expectations to identify candidates for positive and balancing selection from a plot of average locus heterozygosity vs. F_{ST} (Beaumont & Nichols 1996; Beaumont & Balding 2004). Simulations were run to independently generate a distribution of F_{ST}, based on 50 000 replicates, for 76 SNPs under an infinite alleles mutation model. The simulation results were then plotted to represent the median and the 95% and 99% quantiles. Loci lying outside these quantiles were outliers putatively under directional or balancing selection, respectively. Simulations were carried out iteratively to avoid an upward bias in quantile ranges (potentially resulting in Type I error for balancing selection) by removing outlier loci above the 95% and 99% quantiles in the initial runs as implemented in LOSITAN.

Tests for association of SNP markers and environment

As tests for outlier loci can often provide false positives or false negatives (e.g., Akey 2009; Hermisson 2009), a three-step process was used to further identify candidate markers in this study. These steps included a spatial analysis method (SAM) to identify initial candidate markers associated with specific environmental variables, followed by univariate linear regression of SNP allele frequencies and environmental variables to identify secondary candidate markers, and finally a verification step to correct for underlying population structure. Details regarding each of these steps are provided later.

To identify SNP markers that were associated with environmental variables, we used the SAM program developed by Joost *et al.* (2008). This approach utilizes general linear models and logistic regression to identify significant associations of habitat characteristics with presence/absence of alleles at genetic markers across all individuals in the study. In the current data set, one allele for each SNP was recoded as present ('1') or absent ('0') for all individual fish. Missing habitat and genotype data were recoded with the non-numerical designation of 'NaN'. Because of the potential of this test to identify false-positive associations, a conservative alpha of 0.01 with Bonferroni correction (final critical value of 0.00146) was used to reduce false positives in the Wald test (as recommended by Joost *et al.* 2008). Markers with *P*-values below the critical value were considered initial candidates for association with respective environmental variables.

To further reduce false positives and provide a more conservative test for association of markers at the population rather than individual level (SAM), allele frequencies of markers that were considered initial candidates from SAM were evaluated for correlation with each environmental variable for the 12 collection sites. We expect that this population-level approach is more conservative because the regression analyses with MAF and temperature only include 12 data points (one per population) as opposed to 499 data points (one per individual) and thus the chance of detecting a significant result is lower. Univariate linear regression analysis with the least-squares method was used to determine the relationship between MAF of each SNP and the environmental variable. A critical level of 0.01 was used to identify significant associations. Initial candidate markers from SAM that were also significant in linear regression tests were considered to be secondary candidate markers. All other markers were considered as putatively neutral for further statistical analyses.

It is well known that underlying population structure can bias association tests and result in false-positive loci that are not actually associated with variables of interest (Lander & Schork 1994; Pritchard & Rosenberg 1999; Excoffier *et al.* 2009). To limit association bias owing to underlying population structure in secondary candidate markers in this study, analyses with STRUCTURE v.2.3.2 (Pritchard *et al.* 2000a; Hubisz *et al.* 2009) and STRAT v.1.1 (Pritchard *et al.* 2000b) were implemented as suggested by Pritchard & Rosenberg (1999). In a recent review by Zhang *et al.* (2008), this STRAT approach has been shown to account for population structure in association studies equally well as other leading methods such as principal components analysis (i.e., Price *et al.* 2006). We followed the procedure for running STRAT with the following steps: (i) candidate loci were identified with regression analyses, (ii) candidate loci and any other significantly linked markers (linkage

disequilibrium with $P < 0.0059$) were removed from the data set, (iii) remaining loci (putatively neutral) were used to run STRUCTURE and select the most likely number of distinct populations (k) from 10 iterations for each potential k value ranging from 1 to 10 (50 000 burnin followed by 100 000 MCMC repetitions), and (iv) ancestry coefficients (mean Q values from CLUMPP; Jakobsson & Rosenberg 2007) and environmental variables for each individual were included in STRAT to test candidate markers for significant association with environmental variables.

Population structure and adaptation

To infer the degree of relatedness between sample collections, pairwise chord distances (Cavalli-Sforza & Edwards 1967) were calculated between all population samples with the POPULATIONS software package (Langella 2001). These genetic distances were then used to construct neighbour-joining trees of sample populations for 1000 bootstrap replicates. A consensus dendrogram was then constructed with the program TREEVIEW (Page 1996). Two dendrograms were constructed, one to represent genetic relationships because of demographic processes with putatively neutral markers and another to evaluate populations potentially under thermal adaptation with candidate SNPs.

To test for differentiation among populations, pairwise values of the variance in allele frequencies among population samples (F_{ST}; Weir & Cockerham 1984) were estimated in GENEPOP with putatively neutral SNPs and separately with candidate temperature SNPs. Approximations of exact tests with MCMC were performed in GENEPOP (1000 batches and 1000 iterations) to determine significance of pairwise genic differentiation between all collections. Significance levels were adjusted for multiple tests with B-Y FDR (Benjamini & Yekutieli 2001; Narum 2006). Differences in pairwise F_{ST} matrices were evaluated to determine whether specific populations had higher adaptive divergence than neutral divergence.

Patterns of isolation by distance among sites were evaluated with Mantel tests using fluvial stream distance and pairwise F_{ST} from SNPs determined to be either putatively neutral or candidate markers. The regression of the pairwise $F_{ST}/(1-F_{ST})$ on geographic distance was used to determine significance of Mantel tests in GENEPOP (Raymond & Rousset 1995). The procedure was modified to test a model of isolation by temperature by substituting pairwise differences in temperature vs. panels of putatively neutral and temperature-associated SNPs and for each matrix in the Mantel tests. Thus, Mantel tests were able to capitalize on the cumulative effect of all candidate markers (multilocus F_{ST}), rather than single loci, to estimate adaptive divergence and evaluate signals of local adaptation because of temperature differences among collections.

Results

Habitat differences and descriptive statistics

Tests of ANOVA revealed significant differences between desert and montane habitats for the following three environmental variables: width ($P = 0.011$), precipitation ($P = 0.00001$) and temperature ($P = 0.002$). The other six environmental variables were not significantly different between the two climates [gradient ($P = 0.170$), specific conductivity ($P = 0.067$), fines ($P = 0.463$), gravel ($P = 0.910$), cobble/boulder ($P = 0.727$) and elevation ($P = 0.879$)]. Daily stream and air temperatures for each site were significantly correlated (all P-values $<1.0 \times 10^{-8}$), with a range of r^2 values from 0.24 to 0.72 across sites (Fig. S1, Supporting Information).

Of 912 tests for deviation from Hardy–Weinberg equilibrium (76 SNPs in 12 populations), there were 10 significant results (BY-FDR critical value of 0.007) that included nine heterozygote deficits and one heterozygote excess. Deviations were stochastically distributed across loci and populations with two deficits in Big Jacks Cr. (Omy_97865-196 and Omy_aromat-280), one deficit in Fawn Cr. (Omy_Ots249-227), three deficits and one excess in Duncan Cr. (Omy_aldB-165, Omy_aromat-280, Omy_Ots208-138, Omy_nkef-308, respectively), one deficit in Whiskey Cr. (Omy_121713-115), two deficits in Johnson Cr. (Omy_121713-115, Omy_LDHB-2_i6) and none in other populations or loci.

Descriptive statistics such as H_E and N_e were estimated with 65 putatively neutral SNPs for each collection and ranged from 0.141 to 0.180 and 18.3 to 406.3, respectively (Table 2). Sites with the lowest estimates of N_e were Duncan Cr. ($N_e = 18.3$) and Fawn Cr. ($N_e = 26.1$) while the highest estimates were from Bennett Cr. ($N_e = 350.9$) and Deer Cr. ($N_e = 406.3$). No significant differences were found among desert and montane collections for H_E ($P = 0.53$) or N_e ($P = 0.38$). However, pairwise exact tests of allele frequencies among collections were all significantly differentiated from one another with or without correction for multiple tests (all P-values were $<1.0 \times 10^{-4}$).

Simulation results from LOSITAN identified a total of eight loci that fell outside the 95% quantile for neutral expectations, with five candidates for divergent selection and three for balancing selection. The five candidates for divergent selection were Omy_97856-196, Omy_aspAT-413, Omy_arp-630, Omy_tlr5-205 and Omy_nkef-308 and the three for balancing selection were Omy_hsp47-86, Omy_hsc71-80 and Omy_NAKATPa3-50. At the more

Table 2 Reclassification of sites by climate categories followed by unbiased heterozygosity (H_E) and estimate of effective size (N_e) with 95% CI

Map no.	Climate category	Collection	Region	Annual avg. max. air temp (°C)*	H_E	N_e	95% CI
5	Cool	McMullen Cr.	Desert	14.2	0.173	31.5	19.1–65.6
8	Cool	Upper Manns Cr.	Montane	12.1	0.156	89.8	37.5–infinite
9	Cool	Fawn Cr.	Montane	12.2	0.141	26.1	15.6–53.3
11	Cool	Whiskey Jack Cr.	Montane	13.0	0.165	63.6	39.0–131
Average of 'cool' populations				12.9	0.159	52.8	—
1	Undetermined	Deer Cr.	Desert	13.8	0.151	406.3	91.4–infinite
7	Undetermined	Johnson Cr.	Montane	13.4	0.166	228.5	90.7–infinite
10	Undetermined	L. Weiser Cr.	Montane	13.3	0.180	99.4	40.1–infinite
12	Undetermined	Keithley Cr.	Montane	13.0	0.167	49.5	22.6–871.8
Average of 'undetermined' populations				13.4	0.166	195.9	—
2	Warm	Big Jacks Cr.	Desert	14.5	0.176	34.8	21.3–71.7
3	Warm	Little Jacks Cr.	Desert	16.8	0.146	133	61.1–2426.1
4	Warm	Duncan Cr.	Desert	15.0	0.153	18.3	14.1–23.6
6	Warm	Bennett Cr.	Desert	14.6	0.148	350.9	44.2–infinite
Average of 'warm' populations				15.2	0.156	134.3	—

*Indicates significant value in ANOVA (air temperature $P = 0.009$).

stringent 99% quantile, only two markers were considered candidates, one each for positive (Omy_aspAT-413) and balancing (Omy_hsc71-80) selection.

Tests for association of SNP markers and environment

Analysis with SAM provided a total of 24 markers that were significant for one or more environmental variable for a total of 61 significant associations (Table S2, Supporting Information). Each of the nine environmental variables were significant with at least one marker (Table S2, Supporting Information), but temperature had the most number of associated markers (17), followed by precipitation (13), specific conductivity (13), elevation (11), fines (3), gradient (1), width (1), gravel (1) and cobble/boulder (1). The 24 markers that were significantly associated with environmental variables were considered initial candidate markers.

Linear regression of population allele frequencies and environmental variables was used as a secondary criterion to screen the initial 24 candidate markers identified from SAM. Of the 61 initial associations, only seven had significant correlation between the specified environmental variable and MAF. Of these seven significant results, five were markers associated with temperature, one marker with precipitation and one marker with specific conductivity. The five loci with allele frequencies significantly correlated with temperature (Fig. 2) were Omy_aldB-165 ($P = 0.0051$), Omy_gdh-271 ($P = 0.0005$), Omy_Ogo4-212 ($P = 0.0008$), Omy_stat3-273 ($P = 0.0089$) and Omy_tlr5-205 ($P = 0.0062$). One of these five markers (Omy_Ogo4-212) was also significantly correlated with specific conductivity ($P = 0.005$). An additional marker was associated with precipitation (Omy_hsf2-146; $P = 0.007$; Fig. 2). In total, seven of the 61 significant associations identified by SAM were also significantly correlated with linear regression of population allele frequencies. Therefore, the six SNPs that accounted for these seven significant associations were considered secondary candidate markers for further verification.

In the third and final verification step for candidate markers, correction for underlying genetic structure was applied as described in the methods section (STRUCTURE/STRAT) with the following panels of markers. A total of 65 putatively neutral SNPs were used to determine population structure in STRUCTURE, removing the six candidate SNPs and five markers linked to them (Omy_113490-15, Omy_aromat-28, Omy_IL17-185, Omy_Ots249-22, Omy_rapd-167). Results from STRUCTURE

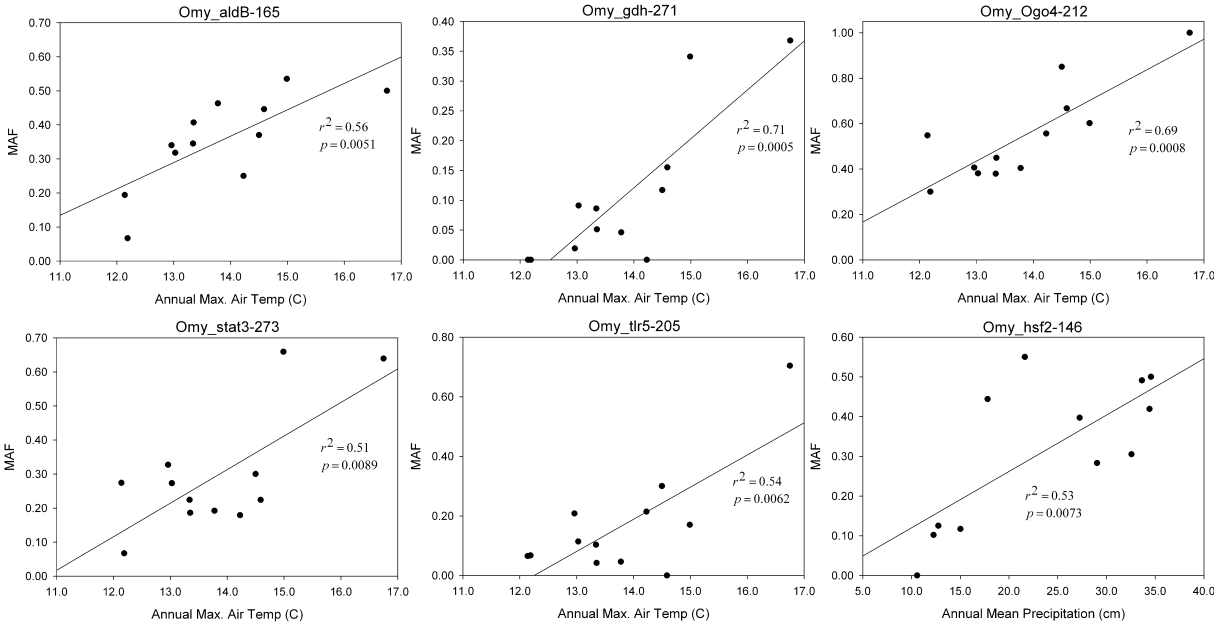

Fig. 2 Correlation of minor allele frequency for five candidate temperature markers and one candidate precipitation marker.

provided support for $k = 5$ as determined by criteria in Evanno *et al.* (2005). Mean ancestry coefficient (Q) and environmental variables (temperature, precipitation and specific conductivity) for each individual were included as input for STRAT analysis to correct for population structure of the six candidate markers. All six candidate markers remained highly significant after correction for genetic structure (all P-values <0.0001) and therefore were considered validated candidate SNPs associated with temperature (Omy_aldB-165, Omy_gdh-271, Omy_Ogo4-212, Omy_stat3-273), temperature and specific conductivity (Omy_Ogo4-212) or precipitation (Omy_hsf2-146). Thus, panels of 65 putatively neutral markers and five temperature-associated markers were used in further analyses. Single loci associated with either precipitation or specific conductivity remained verified candidates but were not tested further.

Population structure and adaptation

Patterns of genetic structure and adaptation were observed when suites of 65 putatively neutral or five temperature-associated markers were examined separately. With the panel of 65 putatively neutral SNP markers, all collections were genetically differentiated from one another as pairwise estimates of F_{ST} were highly significant ($P < 0.0001$) among all population combinations (Table 3, lower matrix). However, desert sites on average had higher pairwise F_{ST} values than montane sites. Results from STRUCTURE analysis with the 65 putatively neutral SNPs further revealed that collections from desert locations were more genetically distinct than those from montane sites. With the panel of five temperature candidate SNPs, the highest divergence was observed between desert and montane comparisons (all highly significant), and least among montane vs. montane collections (7 of 15 significant), while all 15 desert vs. desert comparisons remained significant (Table 3, upper matrix). The two matrices were highly correlated (Mantel $P = 0.001$), but 11 of 65 comparisons had at least twofold greater F_{ST} for the candidate than the neutral markers. These 11 comparisons primarily involved one desert population (Little Jacks Cr.) and one montane population (Fawn Cr.; Table 3) that represented the two extreme ends of temperature variation of the 12 collections (Table 1) at 16.8 and 12.2 °C, respectively. Little Jacks Cr. had ≥ twofold higher candidate F_{ST} in four montane and one desert populations, and Fawn Cr. had ≥ twofold higher candidate F_{ST} in four desert and two montane populations (Table 3) as would be expected if adaptive divergence was greater than neutral divergence between populations occupying differing climates.

Neighbour-joining dendrograms with each suite of markers provided differing perspectives regarding genetic similarity of populations included in this study. With the panel of 65 putatively neutral markers, there was no clear evidence for genetic structure of tributary collections within the same river system except the two sites in the Boise River and three sites from the Bruneau River (Fig. 3a). Further, bootstrap support was below 50% for all clusters (except the two Boise River sites),

Table 3 Pairwise F_{ST} with 65 putatively neutral markers below diagonal, and five temperature-associated SNPs above the diagonal

	Deer Cr.	Big Jacks Cr.	Little Jacks Cr.	Duncan Cr.	McMullen Cr.	Bennett Cr.	Johnson Cr.	Upper Manns Cr.	Fawn Cr.	L. Weiser Cr.	Whiskey Jack Cr.	Keithley Cr.
	1	2	3	4	5	6	7	8	9	10	11	12
Deer Cr.	—	0.149	0.387^{2X}	0.143	0.050	0.041	-0.006^{NS}	0.055	0.120	0.000^{NS}	0.027	0.006^{NS}
Big Jacks Cr.	0.132	—	0.166	0.087^{2X}	0.061	0.062	0.130	0.096	0.279^{2X}	0.126	0.103	0.114
Little Jacks Cr.	0.193	0.104	—	0.153	0.319	0.312	0.382^{2X}	0.362	0.504^{2X}	0.357^{2X}	0.315	0.339
Duncan Cr.	0.144	0.042	0.166	—	0.150	0.099	0.147	0.150	0.298^{2X}	0.129	0.104	0.112
McMullen Cr.	0.113	0.153	0.236	0.187	—	0.058	0.031	0.006^{NS}	0.089	0.022^{NS}	0.018^{NS}	0.019^{NS}
Bennett Cr.	0.090	0.123	0.221	0.157	0.060	—	0.028^{NS}	0.053	0.208^{2X}	0.049	0.073	0.049
Johnson Cr.	0.060	0.083	0.155	0.110	0.078	0.084	—	0.032^{NS}	0.100	-0.003^{NS}	0.024	0.001^{NS}
Upper Manns Cr.	0.068	0.090	0.188	0.091	0.067	0.060	0.045	—	0.085^{2X}	0.025^{NS}	0.027^{NS}	0.015
Fawn Cr.	0.125	0.085	0.189	0.103	0.090	0.067	0.079	0.043	—	0.074	0.099	0.078^{2X}
L. Weiser Cr.	0.050	0.064	0.165	0.077	0.059	0.055	0.037	0.025	0.044	—	0.003^{NS}	-0.017^{NS}
Whiskey Jack Cr.	0.095	0.093	0.187	0.122	0.090	0.092	0.049	0.065	0.068	0.070	—	-0.005^{NS}
Keithley Cr.	0.078	0.061	0.173	0.096	0.074	0.030	0.046	0.031	0.035	0.033	0.061	—

SNP, single-nucleotide polymorphism.
All pairwise comparisons were significant at corrected critical value of 0.01 except those labelled with 'NS' (not significant). Differences in upper vs. lower F_{ST} matrices that were ≥2.0 are indicated by '2X'. The first six collections were from desert regions, and the last six collections were from montane regions.

indicating genetic similarity among collections and limited divergence. Conversely, clear patterns of climate adaptation were observed with the five temperature-associated markers (Fig. 3b). Clustering patterns from the neighbour-joining tree with the five temperature-associated SNPs indicated that some sites may be adapted to warm or cool climates, but four sites were considered undetermined (Fig. 3b, Table 2). Of note, one collection (McMullen Cr.) that was originally classified as a desert population clustered with cool populations, and another desert collection (Deer Cr.) was undetermined. Three collections originally classified in the montane regime clustered as cool, but three others were undetermined in the neighbour-joining tree. When reclassified into climate categories, air temperature was significant among groups, but differences in N_e and H_E were not significant (Table 2).

Results with the five temperature-associated SNPs also revealed that cluster patterns in the neighbour-joining tree were consistent with results from pairwise F_{ST} with these five markers (Table 3), as both analyses demonstrated genetic similarity of sites within each group of cool- or warm-adapted or undetermined populations. Population pairs within cool and undetermined groups were not well differentiated (15 of 28 comparisons were not significant), while the warm-adapted populations remained significantly different from one another and cool/undetermined collections in all but one pairwise comparison. Of the warm-adapted populations, Bennett Cr. had the least differentiation from undetermined sites. Results in STRUCTURE with the five temperature-associated SNPs had $k = 2$, with three desert populations (Big Jacks Cr., Little Jacks Cr. and Duncan Cr.) containing a majority of membership of one group, and all other populations having majority membership in the other group.

There was no significant support for an isolation-by-distance model (Fig. 4a) among all collections with 65 putatively neutral SNPs ($r^2 = 0.02$, Mantel test $P = 0.49$). However, when desert populations were removed, montane populations had a significant isolation-by-distance relationship ($r^2 = 0.25$, Mantel test $P = 0.012$). Isolation by distance was not significant with the five temperature candidate markers ($r^2 = 0.006$, Mantel test $P = 0.14$). These weak relationships of isolation by distance were in contrast to the strong isolation-by-temperature relationship observed among collections with the five candidate SNPs associated with temperature (Fig. 4b; $r^2 = 0.69$, Mantel test $P < 0.00001$). The isolation-by-temperature relationship remained significant with all 18 temperature SNPs that were initially identified by SAM ($r^2 = 0.53$, Mantel test $P = 0.008$), but not with the remaining 58 SNPs ($r^2 = 0.01$, Mantel test $P = 0.14$). Single marker relationships with other envi-

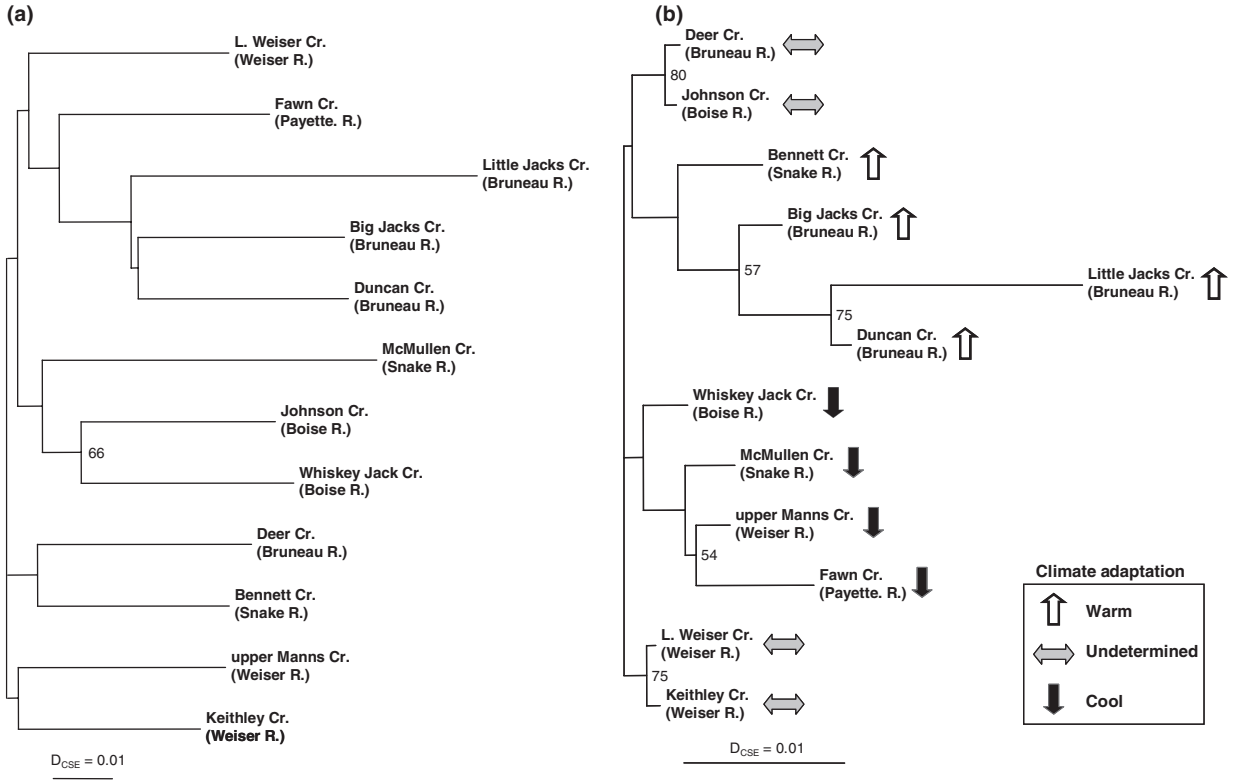

Fig. 3 Neighbour-joining diagram of 12 collections of redband trout (*Oncorhynchus mykiss*) as determined by (a) panel of 65 putatively neutral single-nucleotide polymorphisms, and (b) panel of five candidate markers associated with temperature. Arrows depict cool- or warm-adapted or undetermined populations as categorized by clustering patterns. Chord distance (Cavalli-Sforza & Edwards 1967) is given at the lower left, and bootstrap values from 1000 iterations that were greater than 50% are shown.

ronmental variables (precipitation and specific conductivity) were not explored because of limited ability to detect population differentiation with one locus.

Discussion

In this study, we demonstrated that environmental factors may limit gene flow and also act as a driving force for local adaptation of redband trout in various climates. Previous studies have established temperature as an environmental variable that shapes genetic structure in natural populations of salmonids (Dionne *et al.* 2008; Narum *et al.* 2008). Here, we found clear evidence for genetic divergence and limited gene flow in desert populations, but redband trout from cooler sites were genetically similar to one another. Climate of desert streams may limit gene flow of redband trout directly through barriers to dispersal such as intermittent stream flow in summer seasons and disconnect among stream systems (Zoellick 1999; Meyer *et al.* 2010). While an isolation-by-distance relationship with neutral markers was not significant across all desert and montane populations, this was likely due to the high genetic differentiation of isolated desert populations in close proximity to one another. In fact, montane populations fit an isolation-by-distance model when desert collections were removed from the analysis with neutral markers. Overall, neutral genetic variation appears to be influenced by a variety of factors including barriers to dispersal (i.e., disconnected desert streams), localized spawning and small population size that are known to influence genetic structure in this species (e.g., Taylor 1995; Heath *et al.* 2002).

Conversely, divergence at candidate loci may be reinforced by local adaptation to environmental conditions (i.e., isolation by adaptation; Nosil *et al.* 2008). In the current study, a pattern of isolation by temperature was evident for loci associated with temperature but not for neutral markers, suggesting that the multilocus panel of candidate markers was successful at identifying adaptive divergence among collections in differing climates. Further, greater levels of adaptive than neutral divergence were apparent in specific populations of redband trout, particularly among those at temperature extremes from other populations in the study. In addition to markers associated with temperature, two other cli-

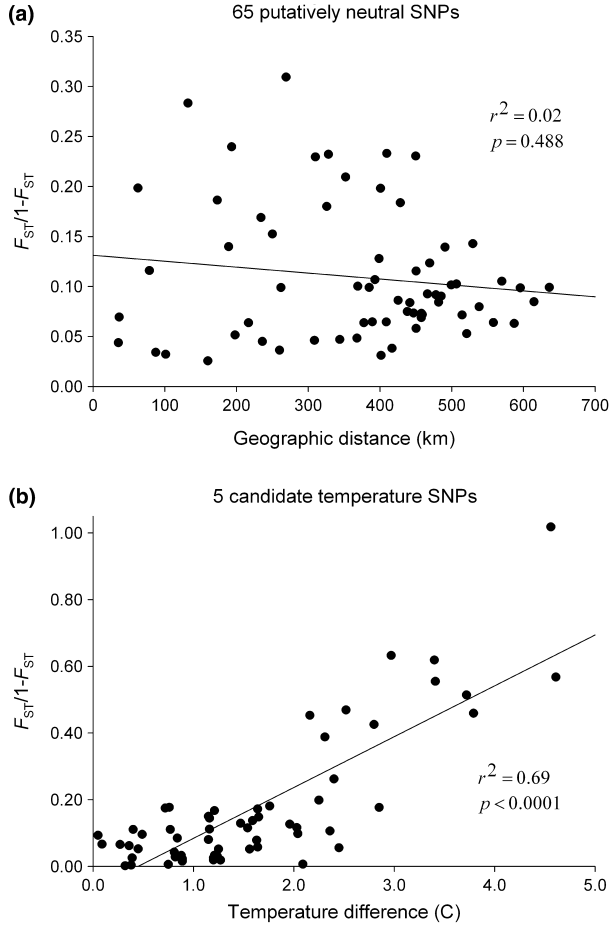

Fig. 4 Patterns of (a) isolation by distance [65 neutral single-nucleotide polymorphisms (SNPs)], and (b) isolation by temperature (five candidate SNPs). Pairwise F_{ST} values are means over loci included in each panel. Geographic distance equals fluvial distance among sites, and temperature equals the difference in annual maximum air temperature of sites.

mate-related variables (precipitation and specific conductivity) were found to be significantly associated with two SNP markers. The strong association of candidate loci with temperature, precipitation and specific conductivity across populations provided support for selection gradients in redband trout related to their environment. These results indicated that selection is acting to influence allele frequencies at specific genes, and redband trout have probably adapted to differing climates throughout their range.

While a single non-synonymous SNP can be responsible for genetic adaptation to a particular environment (e.g., Hoekstra *et al.* 2006), many traits are polygenic. Selection for traits such as thermal tolerance in salmonids may encompass a wide variety of biological pathways such as development rate (Hendry *et al.* 1998), immune response to specific pathogens (Dionne *et al.* 2007), metabolism (Rodnick *et al.* 2004) and stress response (Fowler *et al.* 2009). Our results suggest that numerous candidate genes are involved in climate adaptation in redband trout, consistent with studies in fish indicating that temperature can affect expression of a variety of genes (Kassahn *et al.* 2007) and life stages (Fowler *et al.* 2009). In our study, 7.9% of all the analysed SNPs were found in genes that have a functional role that could potentially be of relevance to thermal adaptation. The evidence for adaptive divergence with multiple candidate SNP markers suggests that many genes are involved in selection and adaptation of redband trout to differing environments.

Markers identified as candidates for selection in this study will require further validation given certain caveats of our approach. Our study included only a limited number of markers because of the lack of genome information for this species, and thus the markers identified as candidates for selection may be part of a selective sweep that would only be identified with a denser scan. A more thorough genome scan would also be likely to produce further candidates than those observed here. Another caveat is that correlation tests of MAF with temperature were based primarily on air instead of water temperatures because of the need for long-term data sets to characterize selective environments over many generations. Daily air temperature was shown to be significantly correlated with daily stream temperatures at all sites in 2009 (Fig. S1, Supporting Information), but long-term stream data would probably improve regression analysis for redband trout. However, long-term stream temperature data sets (~20–30 years) that better capture oscillations in climate are rare, and we have demonstrated that air temperature may serve as an adequate proxy when water temperature data is limited. This relationship is consistent with studies that have specifically examined correlation of air and water temperatures (e.g., Morrill *et al.* 2005).

Adequate characterization of environmental variables is an important but challenging task for genetic association studies in natural populations. We initially classified each site in our study as desert or montane based on geographic location of each stream in either high desert plains or mountain regions. However, further analyses suggested that a priori classification of each site into these categories may not accurately reflect the specific environment that redband trout inhabit and that more fine-scale evaluation of habitat may be needed. Warm and cool categories of adaptation were observed for redband trout that did not always correspond to their initial climate classification (desert or montane) based on stream location. This may be because of limited adaptive variation or complex population history, but it may also be representative of utilization of fine-scale environments. For example, fish

may traverse different areas within a stream because headwaters at higher elevations may be located in cooler habitat than lower segments. Further, upwelling from groundwater sources are common and may create thermal refuges for fish and other aquatic organisms (e.g., Ebersole *et al.* 2001; Tate *et al.* 2007). Variability in natural environments is inherent as seasonal and annual changes occur with climate-related factors (e.g., temperature and precipitation), and human-related disturbances create difficulty in evaluating habitat over long-term periods. In some cases, inter-related environmental variables may benefit studies because one or more factors may be identified as significant when long-term variation cannot be fully realized. While characterization of local adaptation with candidate markers may be a useful tool to determine biological differences (i.e., adaptive variation) of redband trout and other species, complexity in natural environments also reinforces the need for validation of genetic and phenotypic associations under controlled environments to confirm adaptive variation.

Candidate markers that were associated with environmental variables are reasonable targets for further study; however, the significant signal may also be because of linkage with genes or markers not included in this study (e.g., Charlesworth *et al.* 1997). We identified six SNPs as highly significant candidates for selection related to environment in populations of redband trout. These six SNPs were located in cis-regulatory (non-coding) regions of genes and thus may be under regulatory control or closely linked with variation in coding regions. Putative gene function for the six candidate markers (Table 4) suggest that they may play roles in local adaptation to climate in traits such as thermal tolerance and disease resistance. However, these may simply be genes closely linked to the actual regions of the genome controlling for these traits. As the markers identified with the F_{ST} outlier approach were not con-

Table 4 Putative gene function of candidate markers associated with temperature, precipitation and conductivity

SNP marker	Gene	Putative function	References
Omy_gdh-271 (temperature)	Glutamate dehydrogenase	An enzyme present in mitochondria of eukaryotes that converts glutamate to α-ketoglutarate, for urea synthesis. Relevant finding: cold adaptation in Antarctic fish	Ciardiello *et al.* (2000)
Omy_stat3-273 (temperature)	Signal transducers and activator of transcription	This protein transmits signals that help response to bacteria and fungi, and regulation of inflammation. Relevant Finding: response to heat stress	Buckley & Somero (2009)
Omy_aldB-165 (temperature)	Aldolase B	A glycolytic enzyme found in the liver and kidney. Relevant finding: thermal acclimation and stress	Huber & Guderley (1993)
Omy_tlr5-205 (temperature)	Toll-like receptor 5	This gene is a member of the Toll-like receptor (TLR) family that plays a fundamental role in pathogen recognition and activation of innate immunity. Relevant finding: immune response	Bilodeau & Waldbieser (2005)
Omy_Ogo4-212 (temperature and conductivity)	Unknown	Unknown; no BLAST similarity	Olsen *et al.* (1998)
Omy_hsf2-146 (precipitation)	Heat-shock factor 2	Regulates the synthesis of heat-shock proteins during differentiation and development. Relevant finding: responsive to erythrocyte differentiation	Airaksinen *et al.* (1998)

SNP, single-nucleotide polymorphism.

sistent or supported by other association methods (SAM, linear regression, STRAT), it was not possible to validate the markers from outlier tests but these loci may warrant further investigation. Only one (Omy_tlr5-205) of the six candidate markers identified in this study was significant with the F_{ST} outlier approach (Beaumont & Nichols 1996) and highlights the potential for false negatives with general F_{ST}-based approaches. Additional loci identified as candidates with the F_{ST} outlier approach may be under climate-independent selection and thus would not be expected to be correlated with variables tested in this study or they may be false positives. Sampling design may have also influenced or confounded results of F_{ST} outlier tests. Further, habitat characteristics that are related to climate may covary, and it was not possible in this study to identify or predict specific phenotypic traits with genotypic data at candidate markers. However, common garden studies under controlled environments may assist with the determination of candidate markers and their association with specific traits such as thermal tolerance, disease resistance, growth and stress response. More extensive sequencing efforts (i.e., pyro and next generation sequencing) and gene expression of candidate regions may also help to elucidate the specific genes and their functional role in adaptation to climate.

Despite these challenges, genome-wide association studies with dense SNP markers offer the potential to predict phenotypes and 'genetic merit' of individuals (Lee *et al.* 2008; Wray *et al.* 2007) or populations (De Roos *et al.* 2009). While these types of methods have been advocated to predict genetic risk in humans (e.g., Morrison *et al.* 2007) and to enhance artificial selection programmes (e.g., Lande & Thompson 1990), they may also have the potential to identify adaptive variation in natural animal populations for ecological and conservation applications. Our study identified six candidate markers in redband trout that were significantly associated with environment, and further evaluations may lead to a larger number of markers to make robust predictions regarding this species' ability to adapt to climate change. Previous studies suggest that climate change may greatly impact seasonal cues in nature (e.g., Bradshaw & Holzapfel 2008) and cause shifts in species distributions and migration patterns (Berthold *et al.* 1992; Bradshaw *et al.* 2004; Hari *et al.* 2006). However, locally adapted reaction norms may be sufficiently plastic to allow for adaptation to different environments if phenotypic and genetic variation exists (e.g., Jensen *et al.* 2008). Thus, monitoring of candidate gene allele frequencies along genetic clines may prove to be effective for quantifying the influence of climate change on natural populations (e.g., Umina *et al.* 2005). Further, the potential to predict genetic adaptability of individuals and populations to changing climate conditions may have profound implications for many species that face extensive anthropogenic disturbances, but more advanced models are needed that address this issue.

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Supporting information

Additional supporting information may be found in the online version of this article:

Table S1 List of 96 SNP assays genotyped

Table S2 List of SNP markers and significantly associated environmental variables identified with SAM analyses

Fig. S1 Correlation of daily air and stream temperatures for each site from May through October 2009. All relationships were significant with $P < 1.0 \times 10^{-8}$. Stream temperatures for Fawn Cr. were not available.

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