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ARTICLE

Patterns of Hybridization of Nonnative Cutthroat Trout and Hatchery Rainbow Trout with Native Redband Trout in the Boise River, Idaho

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Abstract

Hybridization is one of the greatest threats to native fishes. Threats from hybridization are particularly important for native trout species as stocking of nonnative trout has been widespread within the ranges of native species, thus increasing the potential for hybridization. While many studies have documented hybridization between native cutthroat trout *Oncorhynchus clarkii* and nonnative rainbow trout *O. mykiss*, fewer have focused on this issue in native rainbow trout despite widespread threats from introductions of both nonnative cutthroat trout and hatchery rainbow trout. Here, we describe the current genetic (i.e., hybridization) status of native redband trout *O. mykiss gairdneri* populations in the upper Boise River, Idaho. Interspecific hybridization was widespread (detected at 14 of the 41 sampled locations), but high levels of hybridization between nonnative cutthroat trout and redband trout were detected in only a few streams. Intraspecific hybridization was considerably more widespread (almost 40% of sampled locations), and several local populations of native redband trout have been almost completely replaced with hatchery coastal rainbow trout *O. mykiss irideus*; other populations exist as hybrid swarms, some are in the process of being actively invaded, and some are maintaining genetic characteristics of native populations. The persistence of some redband trout populations with high genetic integrity provides some opportunity to conserve native genomes, but our findings also highlight the complex decisions facing managers today. Effective management strategies in this system may include analysis of the specific attributes of each site and population to evaluate the relative risks posed by isolation versus maintaining connectivity, identifying potential sites for control or eradication of nonnative trout, and long-term monitoring of the genetic integrity of remaining redband trout populations to track changes in their status.

Hybridization with nonnative species or domesticated lineages of the same species (e.g., hatchery-propagated individuals) has become one of the greatest threats to the persistence of native fish (Allendorf et al. 2001; Utter 2004), which are particularly prone to crossing because of their natural mating behaviors (e.g., external fertilization) and the extensive introductions of fish species globally (Rahel 2000; Casal 2006; Gozlan et al. 2010). Hybridization may not always lead to viable or fertile offspring, but even so the wasted reproductive effort can contribute to declines in native populations (Allendorf et al. 2001). If hybrids are reproductively successful, then native and nonnative genomes can become intermixed, or introgressed. Introgression can erode advantageous local adaptations, disrupt coadapted gene complexes, or both, thus causing reduced fitness of hybrid individuals (Araki et al. 2007; McClelland and Naish 2007; Chilcote et al. 2011). In trout, introgression can persist and spread readily (Allendorf et al. 2004; Muhlfeld et al. 2009a), potentially leading to the replacement of native

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populations by hybrid swarms and even to genetic extinction (Rhymer and Simberloff 1996; Epifanio and Philipp 2001).

In the western USA, cutthroat trout *Oncorhynchus clarkii* and rainbow trout *O. mykiss* are not fully reproductively isolated and they readily hybridize, both naturally where they co-occur and as a consequence of human-mediated introductions and invasions outside their historical ranges (Behnke 1992; Kozfkay et al. 2007). Characterization of hybridization between these species has focused primarily on the threat of nonnative rainbow trout introgression with various native subspecies of cutthroat trout (Rubidge et al. 2001; Peacock and Kirchoff 2004; Muhlfeld et al. 2009b; Rasmussen et al. 2010). This has been a major factor in the decline of most extant forms and in the extinction of two cutthroat trout subspecies (Behnke 1992). Comparably little attention has been paid to hybridization in native rainbow trout despite widespread threats from introduced cutthroat trout and hatchery rainbow trout (but see Matala et al. 2008; Simmons et al. 2010; Kozfkay et al. 2011).

Indigenous rainbow trout east of the Cascade Mountains comprise a polyphyletic group commonly referred to as redband trout *O. mykiss gairdneri* (Behnke 1992; Currens et al. 2009; Blankenship et al. 2011). Like most interior western trout, inland redband trout populations have suffered widespread declines and extirpations due to habitat fragmentation, habitat degradation, and the introduction of hatchery trout (Thurow et al. 1997, 2007; Currens et al. 2009). For redband trout, the hybridization threat is posed by nonnative cutthroat trout and by hatchery rainbow trout of largely coastal origin (coastal rainbow trout *O. mykiss irideus*; Behnke 1992). Here, we characterize patterns of hybridization between the native inland redband trout and the nonnative cutthroat trout and coastal rainbow trout in the upper Boise River basin, Idaho. We evaluate patterns of inter- and intraspecific hybridization in the context of stocking history and the influences of barriers to dispersal of nonnative trout. We demonstrate the utility of genetic approaches for rapid characterization of both inter- and intraspecific hybridization across a large landscape, and we discuss implications for monitoring and management to protect and restore inland redband trout.

METHODS

Study area.—The upper Boise River basin (including the North Fork, Middle Fork, and South Fork Boise rivers; Figure 1) drains approximately 5,700 km^2 and was isolated from the lower Boise River by the construction of Arrowrock Dam in 1915 (Rieman and McIntyre 1995). Elevations of stream habitat range from 1,000 to 2,500 m above sea level (Rieman and McIntyre 1995). Higher elevations encompass relatively natural (yet disturbance-prone) habitats mixed with areas degraded by fire suppression, a dense road network, and numerous culverts that are barriers to fish passage (Dunham et al. 2007; Neville et al. 2009). Lower-elevation, main-stem rivers have been highly influenced by dams, reservoirs, and fish stocking. Construction of Anderson Ranch Dam in 1950 isolated the upper South Fork Boise River from the rest of the system. Save for the inclusion of one tributary to Anderson Ranch Reservoir, this upper portion of the South Fork Boise River is not discussed further here and we focus exclusively on the lower South Fork Boise River below Anderson Ranch Dam (Figure 1). Physical connectivity between the South Fork Boise River below Anderson Ranch Dam and the North Fork and Middle Fork Boise rivers is still available through Arrowrock Reservoir (Figure 1). Aside from redband trout, the bull trout *Salvelinus confluentus* is the only native trout. Extensive stocking of nonnative salmon *Oncorhynchus* spp., brook trout *S. fontinalis*, coastal rainbow trout, and cutthroat trout has occurred throughout the last century, with unknown impacts (but see Rieman et al. 2006; Neville et al. 2009).

Study rationale.—This study capitalizes on genotypes from 36 samples of inland redband trout populations across the upper Boise River from a previous study on impacts of wildfire disturbance and culvert barriers (Neville et al. 2009) and combines them with newly obtained genotypes from samples from the South Fork Boise River and its adjacent tributaries (Figure 1). The latter redband trout populations were originally sampled to evaluate the potential for migratory connectivity between the South Fork Boise River and headwater tributaries throughout the upper Boise River, a migratory pattern documented previously in bull trout (Monnot et al. 2008). However, initial results on patterns of hybridization with coastal rainbow trout in the South Fork Boise River prompted analysis of all samples in combination to characterize the influence of coastal rainbow trout and cutthroat trout throughout the watershed.

Fish sampling and tissue collections.—Thirty-six populations were sampled via electrofishing in summer 2004, as previously described by Neville et al. (2009). Briefly, sampling consisted of a single upstream electrofishing pass beginning either above a culvert barrier or at least 300 m above the confluence of the tributary with the Boise River. To ensure collection of a representative population sample and prevent biasing genetic information towards family groups (Hansen et al. 1997), sampling within each tributary was spread out geographically (i.e., collecting from multiple stretches of stream separated by several hundreds of meters) and care was taken to avoid young-of-the-year fish whenever possible. In May 2008, four tributaries flowing directly in to the South Fork Boise River (Rock, Pierce, Dixie, and Granite creeks; Figure 1) were sampled similarly by the U.S. Forest Service. An additional sample was collected from a large section of the South Fork Boise River by angling the upper reach (from Anderson Ranch Dam to the confluence of Rock Creek; Figure 1) in July 2008 and by boat electrofishing the lower reach (below Rock Creek) in October 2008. Small fin clips were collected from all fish and either suspended in a 95% solution of ethanol or desiccated in paper coin envelopes for later use. Additionally, tissue samples of rainbow trout from six hatcheries documented to be the major sources of introductions throughout Idaho were obtained from the Idaho Department of Fish and Game (IDFG) to represent the diversity of nonnative

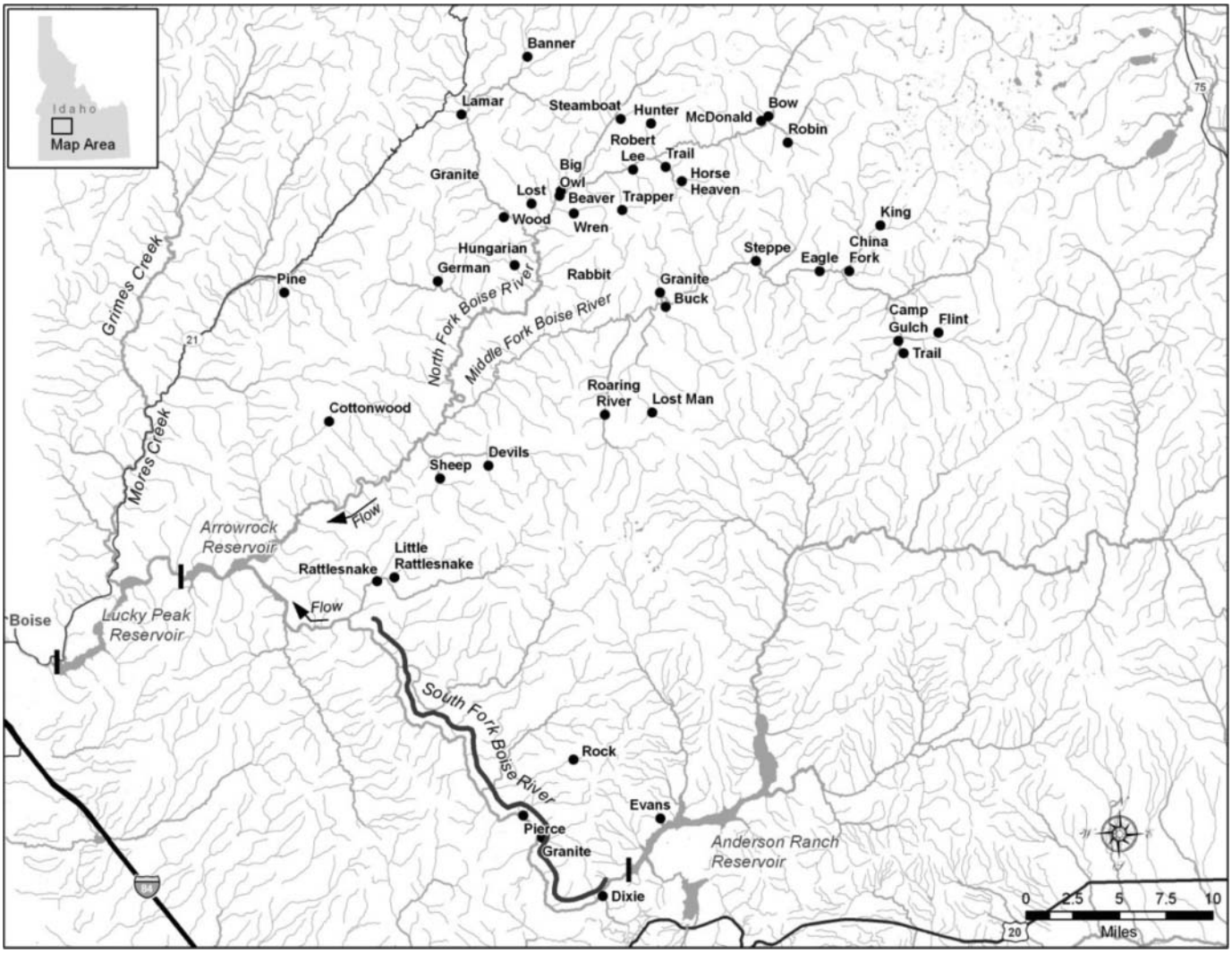

FIGURE 1. Map of the upper Boise River basin, Idaho, including tributaries where redband trout populations were sampled (see Table 1). The North Fork and Middle Fork Boise rivers are connected to the lower South Fork Boise River through Arrowrock Reservoir; Anderson Ranch Dam isolates the lower South Fork Boise River from Anderson Ranch Reservoir and from the upper South Fork Boise River.

rainbow trout genotypes that are likely to be present in the upper Boise River (C. Kozfkay, IDFG, personal communication; Table 1). These samples represented the following hatchery strains or hatchery mixtures: Fish Lake, Hayspur × Kamloops, Mount Lassen × Donaldson, Mount Lassen × Hildebrand, Mount Whitney, and Shepherd of the Hills (see Kozfkay et al. 2011). All are of coastal rainbow trout origin except for the Hayspur × Kamloops hatchery strain, which was established with both coastal rainbow trout and inland redband trout.

Cutthroat trout and coastal rainbow trout stocking history.—Records of fish stocking in the upper Boise River were provided by IDFG and document stocking as early as 1913. Early state records are incomplete, with little or no geographic information, and thus a description of stocking is provided here for background but was not used for statistical analyses of factors such as distance from stocking source or numbers of individuals stocked, as has been achieved by others (e.g., see Muhlfeld et al. 2009b; Bennett et al. 2010). Two subspecies of cutthroat trout were introduced into the Boise River basin historically: pre-1980s stocking used Yellowstone cutthroat trout *O. clarkii bouvieri*, while later stocking used westslope cutthroat trout *O. clarkii lewisi* (M. Campbell, IDFG, personal communication). The only verifiable record of cutthroat trout stocking directly into a tributary sample site was in Rattlesnake Creek in 1940, which was identifiable by its county. Other streams with the same names as our sample sites also were stocked with cutthroat trout in the earlier part of the 20th century (e.g., "Trail," "Trapper," "Devils," and "Cottonwood" creeks are mentioned in the historical records), but there was no information on where these streams were in Idaho to verify their correspondence to

TABLE 1. Characteristics of redband trout samples collected from the upper Boise River, Idaho, and samples of hatchery coastal rainbow trout from six strains that were historically stocked into the system. Where relevant, the major body of water for each population and whether or not the population was isolated by a culvert barrier (Culv: N = no, Y = yes) are listed; *N* is the sample size before removal of individuals with cutthroat trout alleles (tallied under "CT"). Also shown are the average (ave) and median (med) level of introgressive hybridization (*I*) with cutthroat trout and the average and median proportional ancestry in the coastal rainbow trout cluster (*Q*) across individuals within each sample; values in bold italics indicate samples where a creek with a similar name was identified in stocking records for the relevant species (i.e., many were not verifiable even by county [see text]; note that there are two Trail creeks in our samples, so both are in bold italics because a Trail Creek was mentioned in the stocking records). Asterisks indicate introgressed samples (average $Q \geq 0.1$) from systems with no record of stocking. The last column specifies whether or not a stream flows from a headwater lake (NA = not applicable; Rmvd = removed).

Body of water or source	Sample site or strain	Culv	*N*	CT	Ave *I*	Med *I*	Ave *Q*	Med *Q*	Lake
Lucky Peak Reservoir	Pine Creek	N	36		***0.000***	***0.000***	***0.423***	***0.363***	No
North Fork Boise River	Banner Creek	N	36		0.000	0.000	***0.107***	***0.068***	No
	Lamar Creek	N	36		0.000	0.000	0.055	0.028	No
	Robin Creek	N	36		0.000	0.000	0.036	0.019	No
	Bow Creek	N	36		***0.000***	***0.000***	0.040	0.027	No
	McDonald Creek	N	35		0.000	0.000	0.050	0.020	No
	Trail Creek	N	36	4	***0.049***	***0.000***	***0.031***	***0.023***	No
	Horse Heaven Creek	N	36		0.000	0.000	0.065	0.026	No
	Hunter Creek	Y	36		0.000	0.000	0.047	0.021	No
	Robert Lee Creek	N	36	14	0.071	0.000	0.050	0.021	No
	Steamboat Creek	N	36		0.000	0.000	0.020	0.016	No
	Trapper Creek	Y	48	17	***0.106***	***0.000***	***0.041***	***0.027***	No
	Big Owl Creek	N	36	5	0.032	0.000	0.174*	0.04*	No
	Beaver Creek	N	36		***0.000***	***0.000***	***0.049***	***0.030***	No
	Wren Creek	N	36		0.000	0.000	0.045	0.022	No
	Lost Creek	Y	30		***0.000***	***0.000***	***0.032***	***0.027***	No
	Wood Creek	N	30		0.000	0.000	0.061	0.035	No
	Hungarian Creek	Y	36	5	0.024	0.000	0.063	0.018	No
	German Creek	N	36		0.000	0.000	0.129*	0.045*	No
Middle Fork Boise River	Flint Creek	N	30		0.000	0.000	0.023	0.016	No
	Trail Creek	N	36		***0.000***	***0.000***	***0.032***	***0.023***	Yes
	Camp Gulch Creek	N	36		0.000	0.000	0.047	0.032	No
	China Fork Creek	N	35	13	0.090	0.000	***0.130***	***0.052***	No
	King Creek	N	36	1	0.006	0.000	0.052	0.024	Yes
	Eagle Creek	Y	36	6	0.028	0.000	0.075	0.032	No
	Steppe Creek	Y	36		0.000	0.000	0.092	0.036	No
	Buck Creek	N	36		***0.000***	***0.000***	***0.157***	***0.050***	No
	Granite Creek	Y	37		0.000	0.000	0.106*	0.063*	No
	Lost Man Creek	Y	36		***0.000***	***0.000***	0.025	0.017	No
	Roaring River Creek	Y	36		0.000	0.000	0.026	0.018	Yes
Boise River	Devils Creek	N	31	2	***0.012***	***0.000***	***0.061***	***0.037***	No
	Sheep Creek	N	36		***0.000***	***0.000***	***0.389***	***0.359***	No
	Cottonwood Creek	N	36	1	***0.005***	***0.000***	***0.570***	***0.633***	No
South Fork Boise River	Little Rattlesnake Creek	N	36	4	0.024	0.000	***0.502***	***0.535***	No
	Rattlesnake Creek	N	45		***0.000***	***0.000***	***0.852***	***0.910***	No
	South Fork Boise River	NA	185		***0.000***	***0.000***	***0.909***	***0.971***	NA
	Rock Creek	Y	34	32	***0.427***	***0.500***	Rmvd[a]	Rmvd[a]	No
	Pierce Creek	Y	33	1	0.002	0.000	0.954*	0.962*	No
	Granite Creek	Y	33		0.000	0.000	0.951*	0.978*	No
	Dixie Creek	N	35	3	0.064	0.000	0.957*	0.978*	Yes
Anderson Ranch Reservoir	Evans Creek	N	28		***0.000***	***0.000***	0.476*	0.5185*	No
Hatchery	Fish Lake	NA	16	NA	NA	NA	0.948	0.975	NA
	Hayspur × Kamloops	NA	15	NA	NA	NA	0.988	0.990	NA
	Mt. Lassen × Donaldson	NA	16	NA	NA	NA	0.983	0.986	NA
	Mt. Lassen × Hildebrand	NA	16	NA	NA	NA	0.989	0.991	NA
	Mount Whitney	NA	15	NA	NA	NA	0.940	0.965	NA
	Shepherd of the Hills	NA	16	NA	NA	NA	0.974	0.984	NA

[a]Rock Creek sample was removed from STRUCTURE analysis of introgression with coastal rainbow trout (see text).

TABLE 2. Polymerase chain reaction (PCR) laboratory protocols for multiplexed loci used to identify redband trout × cutthroat trout hybrids in the Boise River basin, Idaho (primer = primer concentration).

PCR	Locus	Reference	Primer (μM)	PCR mix	Thermal protocol
Hybrid multiplex	*OMM55*	Ostberg and Rodriguez 2004	0.2	Qiagen MP[a]	95°C for 15 min; 34 cycles of 95°C (30 s), 57°C (1.5 min), and 72°C (30 s); 30 min at 62°C[b]
	OCC38		0.2		
	OCC37		0.2		
	OCC34		0.2		
	OCC42		0.2		
	OCC35		0.1		
	OCC36		0.4		

[a]Qiagen Multiplex Mix (commercial) with 1 unit of HotStart DNA polymerase and 3-mM $MgCl_2$ at pH 8.7 was used for all loci.
[b]Thermal protocol was the same for all loci.

sample sites (see Table 1). Cutthroat trout were stocked in the main-stem Middle Fork and North Fork Boise rivers in 1940 alone, and repeatedly in the South Fork Boise River up until 1967. After 1967, pure cutthroat trout were continually stocked in (unverifiable) headwater lakes, but only hybrid cutthroat trout × coastal rainbow trout were stocked in rivers, tributaries, or reservoirs. Stocking of these hybrids occurred only in the 1990s and not in our sample sites but did occur in areas with direct connection to sample sites (Mores Creek, Grimes Creek, North Fork Boise River, Middle Fork Boise River, and Arrowrock Reservoir; Figure 1). Records of coastal rainbow trout stocking show extensive introductions over many decades into Arrowrock and Anderson Ranch reservoirs and multiple unspecified sites in the main-stem North Fork, Middle Fork, and South Fork Boise rivers. Stocking of coastal rainbow trout in the South Fork Boise River below Anderson Ranch Dam was ceased in 1978. Fifteen tributary sample sites were also likely stocked with coastal rainbow trout, but many were not verifiable (including Rock Creek, which ultimately was removed from analyses of hybridization with coastal rainbow trout; see below and Table 1). Many headwater lakes across the basin were stocked with coastal rainbow trout, but locations could not be verified for this study (see Table 1 for tributaries with lakes). Since 2001, all hatchery rainbow trout planted in Idaho have been sterile triploids (Meyer et al. 2010).

Laboratory processing.—Total genomic DNA was extracted by using DNeasy extraction kits (Qiagen, Valencia, California) and diluted to 5 ng/μL after quantification with fluorometry. Polymerase chain reactions were performed for two distinct sets of markers (described below) in 15-μL reactions using 20 ng of DNA. For all markers, fragment sizing was performed by the Nevada Genomics Center (Reno) with a Prism 3730 DNA Analyzer (Applied Biosystems, Foster City, California), and individuals were genotyped manually with GeneMapper version 3.0 (Applied Biosystems; see Neville et al. 2009 for additional details on all laboratory methods).

Inland redband trout hybridization with cutthroat trout.—The 36 samples of redband trout populations from the upper Boise River were previously evaluated for hybridization with cutthroat trout by using seven biparental, co-dominant markers that were diagnostic for rainbow trout (*O. mykiss* of all subspecies) or cutthroat trout (Ostberg and Rodriguez 2004; see Neville et al. 2009). The more recently collected samples from the South Fork Boise River and its adjacent tributaries were similarly screened for hybridization with cutthroat trout by using this set of markers (Table 2). For rainbow trout or cutthroat trout uninfluenced by introgression, each marker amplifies an allele specific to that species, while rainbow trout × cutthroat trout hybrids possess a heterozygous genotype. The degree of introgressive hybridization (*I*; ranging from 0 to 1) was calculated for each individual by summing the number of cutthroat trout alleles observed and dividing this sum by the total number of alleles amplified (up to 14). We averaged *I* across all individuals for each population to summarize overall levels of hybridization. Each hybrid individual was also classified as follows (see Rubidge and Taylor 2005): individuals that were heterozygous at all loci were classified as F_1 hybrids (the product of a rainbow trout and a cutthroat trout), those that were homozygous at one or more loci for only one parental species were classified as backcrosses (the product of a hybrid and a parental type), and those that had at least one locus that was homozygous for each parent species were classified as post-F_1-generation or F_n hybrids (the product of a hybrid and a hybrid). The markers used here should have high power to detect hybridization in most cases; for example, for seven markers, the probability of mistakenly categorizing a first-generation backcrossed individual as a pure parental type is 0.0078 (Boecklen and Howard 1997). However, even seven markers represent only a small portion of the genome and were originally evaluated in only a sample of the parental subspecies (Ostberg and Rodriguez 2004); this approach may therefore tend to underestimate introgression, particularly where multiple generations of backcrossing has taken place, and may tend to misclassify hybrid types in some cases (e.g., an F_1 could prove to be a backcross if more markers are evaluated).

Inland redband trout hybridization with coastal rainbow trout.—Markers that were diagnostic for inland redband trout

versus coastal rainbow trout were not available. Therefore, we used a clustering analysis and genetic assignment tests to assign individuals genotyped at neutral microsatellite markers to an inland redband trout, coastal rainbow trout, or hybrid origin. Others have demonstrated this to be a powerful method for discriminating closely related species (e.g., Boyer et al. 2008 found admixture identified by this method was 99% correlated with estimates based on seven diagnostic markers) and even for evaluating the impact of hatchery fish on wild fish of the same species (see Hansen et al. 2001a; Simmons et al. 2010; Kozfkay et al. 2011). The 36 samples from the upper Boise River had been genotyped previously at 15 microsatellite loci (Neville et al. 2009); 10 of these loci were used in the present study, and thus the newly obtained samples from the South Fork Boise River, its tributaries, and the hatcheries were genotyped for this subset of loci (loci and laboratory protocols are given in Table 3). The Bayesian clustering algorithm in STRUCTURE (Pritchard et al. 2000) determines population structure by using information from individual genotypes to create clusters (of unknown number k) that maximize the fit to theoretically expected patterns of Hardy–Weinberg equilibrium and linkage equilibrium, while estimating the proportional ancestry (Q) of individuals in each cluster. Here, we followed a well-established method of forcing STRUCTURE to characterize two clusters ($k = 2$; see also Hansen et al. 2001b; Boyer et al. 2008; Sanz et al. 2009; Simmons et al. 2010; Kozfkay et al. 2011), assumed to capture a marked genetic distinction between redband trout of interior origin and hatchery rainbow trout of largely coastal origin (Currens et al. 2009; Blankenship et al. 2011; Kozfkay et al. 2011). The Q of each individual was estimated; Q ranged from 0 (inland redband trout-type) to 1 (coastal rainbow trout-type), and intermediate values indicated various levels of introgression. Because of uncertain stocking records, we did not have samples from reference redband trout populations that are truly known to be unaffected by hybridization with coastal rainbow trout, but the Bayesian clustering method used here can be highly effective even without reference samples (Hansen et al. 2001b; Boyer et al. 2008). The level of hybridization in each sample was summarized by averaging the individual Q-values. We used an average Q of 10% in the coastal rainbow trout cluster to define hybridization, a threshold that has proved most efficient for identifying hybrids and hybrid populations based on simulations and empirical data for a similar number of microsatellites (Sanz et al. 2009; see also Vaha and Primmer 2006). Four separate simulations were performed by using a burn-in length of 100,000 iterations and 100,000 Markov chain Monte Carlo replicates for each run. The run with the highest log-likelihood was used for evaluating hybridization. Results

TABLE 3. Polymerase chain reaction (PCR) laboratory protocols for microsatellite loci used to genotype redband trout sampled in the Boise River basin, Idaho, and coastal rainbow trout representing six hatchery strains (primer = primer concentration). Several sets of loci could be combined in separate multiplex PCRs (multiplexes 1–3) as indicated.

PCR	Locus	Reference	Primer (μM)	PCR mix	Thermal protocol
Multiplex 1	*OMM1286*	Rexroad and Palti 2003	0.15	Qiagen MP[a]	95°C for 15 min; 34 cycles of 95°C (30 s), 56°C (1.5 min), and 72°C (30 s); 30 min at 62°C
	OMM1295	Rexroad and Palti 2003	0.05		
	OMM1178	Rexroad and Palti 2003	0.1		
Multiplex 2	*OCH20*	Robinson et al. 2009	0.1	Qiagen MP[a]	95°C for 15 min; 34 cycles of 95°C (30 s), 62°C (1.5 min), and 72° (30 s); 30 min at 62°C
	OMM1220	Rexroad and Palti 2003	0.04		
	OMM1235	Rexroad and Palti 2003	0.1		
	OMM1236	Rexroad and Palti 2003	0.2		
Multiplex 3	*OCH9*	Peacock et al. 2004	0.06	Qiagen MP[a]	95°C for 15 min; 25 cycles of 95°C (30 s), 67–52°C touchdown (1.5 min), and 72°C (30 s); 10 cycles of 95°C (30 s), 54°C (1.5 min), and 72°C (30 s); 30 min at 62°C
	OCH10	Peacock et al. 2004	0.12		
Single locus	*OMM1173*	Rexroad and Palti 2003	0.2	Single[b]	95°C for 5 min; 36 cycles of 95°C (30 s), 67°C (30 s), and 72°C (30 s); 30 min at 72°C

[a]Qiagen Multiplex Mix (commercial) with 1 unit of HotStart DNA polymerase and 3-mM $MgCl_2$ at pH 8.7 was used for all loci except *OMM1173*.
[b]Single-locus PCR with 1× buffer, 3.5-mM $MgCl_2$, 0.83-mM deoxynucleotide triphosphates, and 1 unit of Titanium *Taq*.

were visualized graphically using distruct software version 1.1 (Rosenberg 2004). Individuals that were identified as hybrids with cutthroat trout based on diagnostic markers were removed from this analysis because they would confuse discrimination between coastal rainbow trout and redband trout clusters.

Relationship between hybridization and isolation.—Physical barriers to dispersal have been demonstrated in other studies to influence hybridization patterns by blocking invasion routes to upstream habitats (Rubidge and Taylor 2005; Bennett and Kershner 2009). The 36 sampling sites from the previous study (Neville et al. 2009) had been selected specifically because they were either connected to larger habitats or isolated only by road culvert barriers (i.e., none were isolated by waterfalls or other types of barrier), which are common in the Boise River system. For all sample sites, culverts potentially blocking fish passage were evaluated as true barriers based on the national inventory and assessment protocol for culverts (see Table 1; Clarkin et al. 2005). Separate Mann–Whitney tests were performed in R version 2.11.1 (R Development Core Team 2009) to determine whether introgression varied with connectivity as indicated by the presence or absence of culvert barriers. The Mann–Whitney test is a nonparametric test of a difference in ranked sums of values in two groups (Zar 1999). The values used here were the population-level average I or Q as well as the median I or Q. Type I error (α) was set at 0.05 for each analysis.

RESULTS

Inland Redband Trout Hybridization with Cutthroat Trout

Of a total of 1,605 redband trout individuals captured, 108 individuals had cutthroat trout alleles (Tables 1, 4; Figure 2a). Surprisingly, no cutthroat trout alleles were found in fish sampled from the South Fork Boise River (the only main-stem river habitat sampled), despite records of repeated stocking. Within sites where cutthroat trout alleles were identified, hybrids comprised a range of 3% (Devils and King creeks) to 89% (Rock Creek) of individuals collected for each sample, and the average I ranged from 0.002 (Pierce Creek) to 0.426 (Rock Creek); only Rock and Trapper creeks had I-values of 0.1 or higher, and both of these creeks potentially had been stocked. Cutthroat trout genes were found in redband trout at 14 sites, 9 of which had no historical record of stocking (Table 1; Figure 2a).

Three sampled individuals were identified as hybrids based on the presence of cutthroat trout alleles but could not be further characterized by hybrid type because of poor amplification (Table 4). One parental-type cutthroat trout was identified in Dixie Creek, a tributary to the South Fork Boise River, and five individuals that were classified as F_1 hybrids were found in two headwater tributaries (Trail and Trapper creeks; Table 4). Five cutthroat trout backcrosses were identified in Rock and Dixie creeks; 18 F_n hybrids were characterized, all of which were found in Rock Creek except for one in Big Owl Creek (Table 4). Rainbow trout or redband trout backcrosses comprised the majority of cutthroat trout hybrids (70%) and were found in all streams with cutthroat trout alleles except Dixie Creek. All individuals with cutthroat trout alleles were removed from STRUCTURE analysis of redband trout–coastal rainbow trout hybridization; because almost all of the individuals sampled in Rock Creek were hybridized, this sample as a whole was dropped from further analyses.

TABLE 4. Distribution of individuals with nonnative cutthroat trout genes across all redband trout samples from the upper Boise River, Idaho; fish were categorized as pure cutthroat trout (CT), first-generation hybrids (F_1), rainbow trout or redband trout backcrosses (RB bc), cutthroat trout backcrosses (CT bc), post-F_1-generation hybrids (F_n), or undetermined due to poor amplification of diagnostic alleles (UN). The total number of hybrids per sample is shown in the rightmost column, and the total number of fish per hybrid category is shown in the bottom row.

Stream	CT	F_1	RB bc	CT bc	F_n	UN	Total
Trail Creek (North Fork Boise River)		3	1				4
Robert Lee Creek			14				14
Trapper Creek		2	15				17
Big Owl Creek			4		1		5
Hungarian Creek			5				5
China Fork Creek			13				13
King Creek			1				1
Eagle Creek			6				6
Devils Creek			2				2
Cottonwood Creek			1				1
Little Rattlesnake Creek			4				4
Rock Creek			9	3	17	3	32
Pierce Creek			1				1
Dixie Creek	1			2			2
Total	1	5	76	5	18	3	108

Inland Redband Trout Hybridization with Coastal Rainbow Trout

The individual Q-values were 99.99% correlated across our four runs, indicating that STRUCTURE easily converged on ancestry assignment for individuals. The average Q-value for the coastal (hatchery) rainbow trout samples ranged from 0.940 to 0.989 in one cluster, suggesting high fidelity of hatchery fish to this single "coastal" cluster and sufficient power to discriminate between coastal rainbow trout and native redband trout genomes (Table 1; Figure 3). Hybridization with coastal rainbow trout was highly variable across field samples; average Q-values ranged from less than 0.03 (e.g., Steamboat, Flint, Lost Man, and Roaring River creeks) to 0.957 (Dixie Creek; Table 1; Figure 3). Thus, some samples had Q-values less than 3% with regard to the coastal rainbow trout cluster and Q-values over 97% for the second, "inland redband trout" cluster. In contrast, 39% percent of all field samples had average Q-values

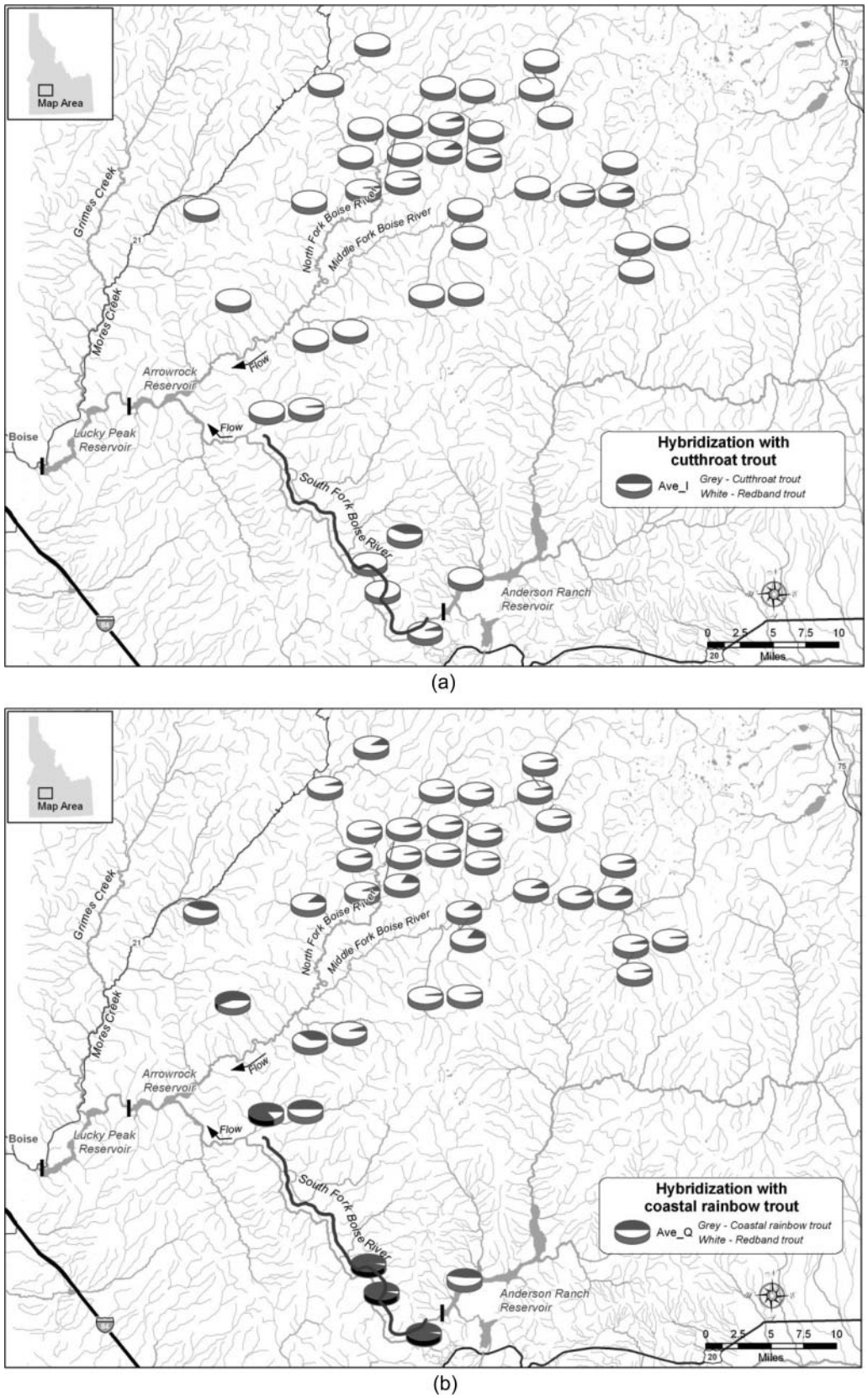

FIGURE 2. Hybridization in redband trout sampled from the upper Boise River, Idaho: **(a)** average introgressive hybridization (ave *I*) with cutthroat trout and **(b)** average proportional ancestry (ave *Q*) in the coastal rainbow trout cluster (i.e., from the STRUCTURE analysis). For some samples, very low levels of introgression (e.g., $\leq$0.005) are not visible on pie charts.

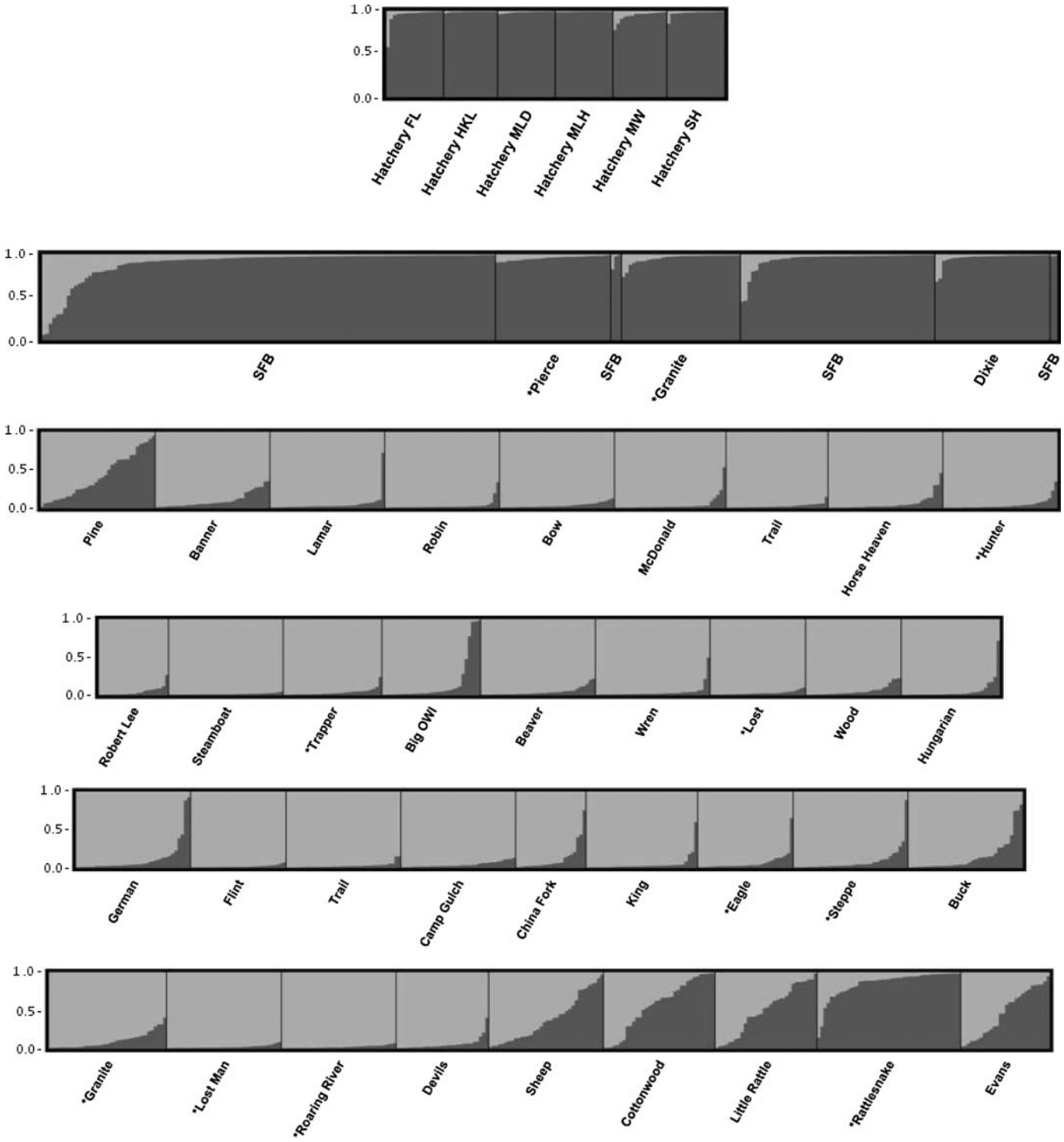

FIGURE 3. STRUCTURE diagram based on two clusters ($k = 2$) for samples from six hatchery strains representing coastal rainbow trout planted in Idaho (FL = Fish Lake; HKL = Hayspur × Kamloops; MLD = Mount Lassen × Donaldson; MLH = Mount Lassen × Hildebrand; MW = Mount Whitney; SH = Shepherd of the Hills) and native redband trout samples from the upper Boise River. Each vertical bar represents an individual fish; dark-gray shading indicates proportional ancestry (Q; scale of 0 to 1) in the coastal rainbow trout cluster, and light-gray shading denotes ancestry in the redband trout cluster. Individuals within samples are ranked by Q-values. Dark vertical lines separate the sample sites (SFB = South Fork Boise River, where several sampling locations are separated by joining tributaries, see Figure 1 and Table 1). Asterisks by sample names indicate tributaries that are isolated by culverts.

of 0.10 or higher for the coastal rainbow trout cluster. These were generally from sites with known or likely stocking events, although seven tributaries that met this threshold had no record of stocking, including many of the tributaries to the South Fork Boise River (Table 1; Figures 2b, 3). The distribution of individual hybrids also varied among populations hybridized with hatchery rainbow trout. For instance, the average Q-value of the Pine Creek sample (0.423) reflected the existence of a "hybrid swarm" at this site, as the sample consisted almost entirely of hybridized individuals. Banner, Buck, Sheep, Cottonwood, Little Rattlesnake, Rattlesnake, and Evans creeks provided other examples of hybrid swarms with varying levels of individual introgression (Figure 3). In contrast, the approximately 17% introgression in Big Owl Creek was generated by a subset of six hybridized individuals (individual $Q = 0.28$–0.98 for the coastal rainbow trout cluster), while many individuals in this sample had very little coastal rainbow trout ancestry ($Q > 0.05$ for the coastal cluster; Figure 3).

Relationship between Hybridization and Isolation

The Mann–Whitney tests showed that redband trout hybridization with cutthroat trout or coastal rainbow trout was not related to the presence or absence of culvert barriers (average I: $\chi^2 = 1.04$, df $= 1$, $P = 0.31$; average Q: $\chi^2 = 0.25$, df $= 1$, $P = 0.62$). The test based on median Q gave similar results ($\chi^2 = 2.245$, df $= 1$, $P = 0.1340$). All samples except for Rock Creek had a median I of 0, and thus there was no variation among connected and isolated sites to test (Table 1).

DISCUSSION

The erosion of genetic integrity through hybridization is one of the greatest threats to the persistence of native trout species worldwide, such that documentation of hybridized populations has become a major conservation research priority (Bennett and Kershner 2009; Sanz et al. 2009). However, identification of hybrid individuals in the field is notoriously difficult, and even sophisticated morphological models have undesirable error rates when introgression levels are low (Smith et al. 1995; Weigel et al. 2002; Seiler et al. 2009). This study demonstrates the utility of genetic data for rapid assessment of inter- and intraspecific hybridization across large landscapes. Although diagnostic markers may be helpful in deciphering the hybridization influence of multiple species at once (as in this study), the clustering approach used here does not require diagnostic markers and in many cases can be applied using the number and type of markers commonly available for population genetic studies (e.g., 6–10 neutral microsatellites; Sanz et al. 2009). Thus, it has broad applicability to any species for which hybridization is of concern, even when hybridization involves a closely related taxon (i.e., subspecies; Hansen et al. 2001a).

For redband trout in particular, accurate characterization of current conservation status has been hindered by a lack of understanding of phylogenetic relationships, historical and current distributions, and rangewide genetic purity in the context of over a century of nonnative trout introductions (Thurow et al. 2007; Blankenship et al. 2011). Our assessment of populations across a large watershed in southern Idaho uncovered a spatially variable footprint of hybridization in native redband trout, and patterns were not always predictable (Boyer et al. 2008; Bennett et al. 2010; Rasmussen et al. 2010). Evaluation of population-average levels of introgression and the distribution of different types of hybrid individuals demonstrates that these fish currently exist in states ranging from genetically intact populations, to actively invaded populations, to hybrid swarms in which the native species has been almost completely replaced by nonnative trout.

Aside from stocking of headwater lakes until 2009, historical cutthroat trout introductions in the Boise River basin occurred in fewer locations and over a shorter period of time than the more widespread, long-term stocking of coastal rainbow trout. Cutthroat trout hybridization was accordingly more limited in degree, since only two redband trout populations were characterized as showing evidence of hybridization with cutthroat trout ($I \geq 0.1$; Table 1). Still, 14 of the 41 Boise River samples contained individuals with cutthroat trout alleles, and nine of these samples were from sites with no record of historical stocking, pointing to possible invasion by cutthroat trout. Conversely, many samples from sites with potential historical stocking had no cutthroat trout alleles (Table 1). One surprising finding was that no cutthroat trout hybrids were detected in the South Fork Boise River despite repeated stocking there until 1967. It is unclear why there would be no detectable lingering impact of this stocking history. One possibility (see Methods) is that our markers failed to identify some hybrids, particularly if multigeneration backcrosses were present, although our power to detect most hybrid types was relatively high. Additionally, this stretch of river has been dramatically altered by humans in recent decades (see below) and was heavily stocked with coastal rainbow trout for at least a decade after the cessation of cutthroat trout stocking; these perturbations may have facilitated the displacement or dilution of the genetic contribution of any established cutthroat trout over time.

Where cutthroat trout hybrids were found, cautious evaluation of the distribution of individual hybrid types (see Methods for a caveat about the power of discrimination among hybrid types) may provide some insight on the etiology of redband trout hybridization with cutthroat trout in the upper Boise River. As mentioned above, stocking of cutthroat trout in the river and reservoir system ceased in 1967 and stocking of rainbow trout × cutthroat trout hybrids occurred at limited locations for a short period in the 1990s. It was therefore not surprising that the most common cutthroat trout hybrids we found were in the form of rainbow trout backcrosses and F_n hybrids; the predominance of these types suggests that at least some of the current cutthroat trout introgression with redband trout is occurring through the persistence and spread of rainbow trout × cutthroat trout hybrids that were possibly created by stocking decades ago. Hybrid individuals (as opposed to parental nonnative individuals) have been found to be primary

vehicles for maintaining and dispersing nonnative genes in several other studies (Hitt et al. 2003; Rubidge and Taylor 2005; Boyer et al. 2008; Bennett and Kershner 2009). In contrast, the finding of some fish with cutthroat trout, F_1 hybrid, or cutthroat trout backcross genotypes suggests that some pure cutthroat trout exist within the tributary system. It is possible that pure cutthroat trout have persisted from the stocking events before 1967 through positive assortative mating with other cutthroat trout and are continuing to generate F_1 hybrids and cutthroat trout backcrosses. This seems somewhat unlikely, but we cannot rule this out as an explanation for the finding of F_1 and cutthroat trout backcross hybrids in Trapper, Trail, and Rock creeks, which may have been stocked with cutthroat trout. Another likely source of cutthroat trout (and coastal rainbow trout) is headwater lakes (see Table 1 for lake locations), many of which were stocked with cutthroat trout until 2009. Stocking records of headwater lakes lacked sufficient geographical information to incorporate them analytically here, but the only other tributary where a parental-type cutthroat trout was found was Dixie Creek, which has a headwater lake. High-elevation lakes are known to be important sources of invasion (Adams et al. 2001). Finally, a threat that has increased in recent decades and that could contribute to the variable patterns observed herein is the illegal introduction of nonnative species (Rahel 2004).

In contrast to redband trout hybridization with cutthroat trout, the influence of coastal rainbow trout on redband trout was more extensive, as samples from almost 40% of sites had hybridized populations ($Q \geq 0.1$). Half of these sites had verified or likely instances of stocking, whereas half of the sites did not, indicating undocumented or illegal introductions or dispersal of hatchery fish and their hybrids. Redband trout in the South Fork Boise River and most of its immediate tributaries have been almost completely replaced by coastal rainbow trout. Although the South Fork Boise River itself has not been stocked with coastal rainbow trout in over 30 years, it flows between two large and heavily stocked reservoirs (Anderson Ranch and Arrowrock reservoirs) that could serve as sources of invasion. Extensive modification of this habitat may also have played a role (Allendorf et al. 2001): dam construction and flow management in the 1940s and 1950s likely caused the near extirpation of native trout in this section of river and may have facilitated their replacement by coastal rainbow trout. Interestingly, two individuals from the South Fork Boise River were characterized here as redband trout ($Q = 0.065$ and 0.077 for the coastal cluster), and it is possible that they represent migrants from elsewhere in the system. A similar migratory pattern has been observed for bull trout in this system (Monnot et al. 2008).

Several recent studies have found correlations between connectivity and hybridization of local trout populations in river networks such that connected sites are more likely to contain introgressed populations (Rubidge and Taylor 2005; Bennett and Kershner 2009). In contrast, we found that connectivity was not consistently related to the degree of hybridization. There are several possible explanations. First, it is possible that some culverts were not absolute barriers (e.g., Burford et al. 2009) as determined by the methods employed herein (Clarkin et al. 2005). Second, periodic failures of culverts, such as breaching in response to extreme flow events, might have allowed some gene flow (Neville et al. 2009). Additionally, since culverts are associated with roads, it is possible by virtue of greater human access to these locations that authorized and unauthorized introductions of nonnative cutthroat trout or coastal rainbow trout are more likely to occur where culverts are present. Invasion from headwater lakes may also erode any barrier effects of culverts, and the lack of a reliable stocking history may have clouded interpretation. Since none of these alternatives is mutually exclusive, it is possible that a mix of these multiple influences affected our ability to observe any simple correlations between hybridization and connectivity.

From a management perspective, the question of how much interspecific hybridization is acceptable for defining populations as representative of the native species has been fiercely debated (Allendorf et al. 2001, 2004, 2005; Campton and Kaeding 2005). Defining the absolute level of hybridization is much more difficult in the case of hybridization among lineages within a species (Sanz et al. 2009). In this study, we found a small degree of genetic overlap between inland redband trout and coastal rainbow trout, evidenced by the fact that coastal rainbow trout had up to 5% assigned ancestry (i.e., Q) in the inland redband trout cluster. Some degree of natural shared ancestry between the two stocks is expected (Currens et al. 2009; Blankenship et al. 2011), and inland redband trout were used historically to develop at least one hatchery stock (see above). In cases where long-term stocking has been pervasive, there is also the concern that hybridization may have affected all remaining populations, thus creating a situation where clustering methods would have reduced discriminatory power (Sanz et al. 2009). Without reference samples from known pure redband trout, we cannot distinguish the influences of these possibilities, but our use of a 10% cutoff for defining introgression with coastal rainbow trout was conservative to account for the fact that our approach was not strictly diagnostic of hybridization and that discriminatory power was relative to the markers and data set used (Vaha and Primmer 2006; Sanz et al. 2009). Still, using fairly simple genetic techniques, we were able to resolve important relative differences among populations in our samples with respect to both inter- and intraspecific hybridization, which could be helpful in prioritizing future management strategies in the basin. For instance, in our evaluation of the average Q-values for redband trout populations in the upper Boise River, many (16) populations met a criterion of having a Q less than or equal to 0.05, and all but three of these populations had no evidence of introgression with cutthroat trout. These stronghold populations may be among the last remaining representatives of the genetic legacy of native redband trout in the Boise River basin, at least among the locations we sampled.

It is still possible to implement relatively simple measures to protect native species such as redband trout. For example,

IDFG has discontinued the stocking of cutthroat trout in the Boise River based in part on the results of earlier work (Neville et al. 2009; Jeffrey Dillon, IDFG, personal communication). The recent practice of stocking only infertile triploid coastal rainbow trout (Meyer et al. 2010) may further reduce threats to native redband trout. With these measures in place, the large established populations of hybridized rainbow trout in the upper Boise River pose a more challenging threat to the genetic integrity of redband trout throughout the basin (e.g., Bennett and Kershner 2009; Horreo and Garcia-Vazquez 2011). Our existing data may be helpful in identifying sources of hybridization that can be controlled—for example, through selective removal of nonnative parental types within streams with such segregation (e.g., Big Owl Creek) or through a more-intense focus on hybrid swarms (Allendorf et al. 2001). Restricting the dispersal of individuals that may be sources of hybridization represents an alternative to direct control but is not a cost-free strategy. For example, in our study system, it is clear that isolated populations are at risk from loss of gene flow tied to movement barriers, such as culverts at stream–road crossings (Neville et al. 2009). A careful analysis of the relative risks posed by isolation versus maintaining connectivity that incorporates specific attributes of each site and population may be informative in weighing management options (e.g., Peterson et al. 2008; Fausch et al. 2009).

The picture of hybridization presented here provides many valuable and immediately useful insights, but it is far from complete. Use of additional genetic markers and further sampling to represent more locations may be justified to establish a truly comprehensive baseline for future genetic monitoring (Schwartz et al. 2006; Vaha and Primmer 2006; Sanz et al. 2009). Historical museum samples collected before hatchery introductions (e.g., Hansen 2002) or collections from remote and inaccessible populations that are reliably known to occur in unstocked systems could provide important references for the genetic characteristics of pure (non-hybridized) native redband trout. Furthermore, whereas documenting the consequences of hybridization is a useful first step, going further to diagnose the causes of hybridization would be particularly informative. This would involve additional efforts to document the historical locations and numbers of nonnative cutthroat trout and hatchery rainbow trout that have been stocked (Bennett et al. 2010) as well as data collections designed specifically to evaluate hypothesized local landscape influences on movement and hybridization (e.g., Rubidge and Taylor 2005; Neville et al. 2006; Muhlfeld et al. 2009b). Such an approach could help to identify populations that are more or less at risk from hybridization as well as specific factors that may contribute to hybridization, including those that could be addressed effectively by future management to protect native redband trout.

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