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ORIGINAL ARTICLE

Estimation of introgression in cutthroat trout populations using microsatellites

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Abstract Introgressive hybridization, mediated by anthropogenic activity, poses a threat to numerous and diverse taxa. The management of introgressed individuals or populations within species of conservation concern is currently the subject of scientific and political debate. We investigate the utility of 10 non-diagnostic microsatellite loci for investigating admixture from introduced Yellowstone cutthroat trout (*Oncorhynchus clarkii bouvieri*) and rainbow trout (*O. mykiss*) within 25 putative Rio Grande cutthroat trout (*O. c. virginalis*) populations. We apply five different approaches (correspondence analysis, maximum-likelihood assignment tests, an admixture estimator based on allele frequencies, an admixture estimator based on coalescent theory and an admixture estimator implementing a Bayesian method) and use two alternative *O. c. virginalis* reference samples. All approaches were capable of identifying one population that consisted entirely of introduced *O. c. bouvieri*, and three out of five approaches enabled us to discriminate those populations with relatively high levels of non-native introgression from those populations with little or none. Actual estimates of admixture coefficients within a test population, varied, however, with the approach and reference sample used. These results have important implications for policies dividing populations into different management categories according to the estimated proportion of non-native genetic material that they contain.

Keywords Conservation · Endangered Species Act · Hybrid · Introgression · *Oncorhynchus clarkii* · Microsatellite

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Introduction

Introgressive hybridization, mediated by human activity, poses a threat to numerous and diverse taxa (Rhymer and Simberloff 1996; Wolf et al. 2001; Jensen et al. 2005). Incorporation of even small amounts of non-native genetic material into the genome could increase the extinction risk of a native population by causing outbreeding depression (Edmands 1999; Montalvo and Ellstrand 2001; Marr et al. 2002; Gilk et al. 2004). Ongoing introgression can cause the population to be replaced by one genetically and phenotypically resembling the non-native form (Huxel 1999). The identification and management of introgressed (admixed) populations within taxa of conservation concern present a number of challenges. Individuals containing non-native genetic material may be morphologically indistinguishable from those containing an intact native genome (Daniels et al. 1998; Weigel et al. 2002; Chan et al. 2006). While admixed populations can pose a hybridization threat to extant pure populations, they may also contain native genetic diversity not represented in those populations (Porath and Nielsen 2003; Peacock and Kirchoff 2004; Campton and Kaeding 2005). Decisions regarding treatment of introgressed individuals or populations are best

made on a case-by-case basis (Allendorf et al. 2001, 2004). However in practice many bodies concerned with the conservation of native taxa might prefer to work with strict guidelines that they can use, for example, to justify decisions in a court of law.

The problem of introgressive hybridization with introduced non-natives is well documented in freshwater fishes, where isolation in different water bodies can allow evolutionary diversification in the absence of intrinsic barriers to gene exchange (Epifanio and Nielsen 2000; Perry et al. 2002; Rosenfield et al. 2004). The issue has been particularly well studied in salmonids, which are threatened by the widespread stocking of hatchery-reared individuals for the purpose of commercial or recreational fishing (Utter 2001). For example, in Europe, marble trout (*Salmo marmoratus*) and geographically distinct lineages of brown trout (*S. trutta*) and Arctic charr (*Salvelinus alpinus*) are at risk from introgression from introduced congeneric hatchery strains (Brunner et al. 1998; Machordom et al. 1999; Hansen et al. 2001; Fumagalli et al. 2002). Similarly, in interior North America, Apache trout (*Oncorhynchus apache*), Gila trout (*O. gilae*), golden trout (*O. aguabonita*), rainbow trout (*O. mykiss*) and all eight extant subspecies of inland cutthroat trout (*O. clarkii*) are threatened by hybridization with various *Oncorhynchus* species stocked as sport fish (Behnke 1992; Kruse et al. 2000; Porath and Nielsen 2003; Wares et al. 2004; Rubidge and Taylor 2005). In response to the problem of introgressive hybridization, management of cutthroat trout in seven U.S. states is currently guided by a Position Paper (Anonymous 2000) which divides populations into management categories according to the proportion of non-native genetic material that they are estimated to contain. The 'Core Conservation' category includes all populations exhibiting < 1% introgression from non-native trout. The 'Conservation' category includes populations that correspond phenotypically to pure native cutthroat trout but which contain more non-native genetic material; most agencies include populations with < 10% introgression within this category. All remaining populations are termed 'Sportfish', and generally managed as non-native trout.

Categorization of populations using such quantitative thresholds necessitates a method to accurately assess admixture levels. In practice the precision of an admixture estimate will depend upon multiple factors, including the sampling design, the type of marker used, and the degree to which the true situation conforms to the assumptions underlying the statistical methodology chosen. The ideal genetic marker for estimation of introgression levels is selectively neutral, biparentally inherited, co-dominant, diagnostic between the taxa of interest at multiple loci, and allows non-lethal sampling. Discovery of such ideal markers, however, may be difficult. For example, the closely related cutthroat trout subspecies *O. c. pleuriticus*, *O. c. stomias* and *O. c. virginalis* are all known to have hybridized with introduced Yellowstone cutthroat trout (*O. c. bouvieri*) within their native range, but diagnostic markers currently available to distinguish between the native and non-native taxa either require lethal sampling (allozymes, Keeler-Foster 2003), are inherited through the maternal line only (mtDNA, J. L. Metcalf, personal communication) or are not co-dominant (Paired Interspersed Nuclear Elements or PINEs, Kanda et al. 2002; Colorado Division of Wildlife unpublished data). A further complication arises where all populations in a taxon's range have potentially been exposed to hybridization, in which case identification of pure populations may be difficult. Even where pure populations can be unambiguously identified, they may not be suitable for reference purposes. Specifically, where gene exchange between populations has been historically limited, the available pure reference sample may be genetically quite different from the native ancestors that contributed to the admixed population under consideration. In such a situation, an admixture estimate may vary with the reference sample used.

Microsatellites have recently become a marker of choice in population genetic studies. Their high mutation rate and limited number of possible allelic states means that taxa frequently exhibit overlapping allele size distributions, and hence microsatellite loci are rarely completely diagnostic between hybridizing populations. However a number of alternative approaches are available to investigate admixture using such highly variable markers (Choisy et al. 2004; Freeman et al. 2004), several of which can be applied to situations where more than two parental taxa are involved. All these approaches are, by necessity, based on simplifying assumptions that are unlikely to be fulfilled in the true situation under consideration, hence different approaches are expected to generate different admixture estimates. In this paper, we examine the efficacy of five of these approaches, applied to data from 10 microsatellite loci, for identifying or quantifying non-native introgression in populations of Rio Grande cutthroat trout, *O. c. virginalis*. Native to the Canadian, Pecos and Rio Grande river drainages of Colorado and New Mexico, *O. c. virginalis* has declined to a fraction of its historic range and currently has a highly fragmented distribution, being primarily restricted to small headwater streams (Pritchard and Cowley 2006). The taxon exhibits substantial

genetic structuring at both the inter-population and inter-drainage level (global F_{st} within the Rio Grande drainage = 0.4, Pritchard and Cowley 2006). Although non-natives are no longer stocked into waters known to contain *O. c. virginalis*, all extant populations have potentially been exposed over the past century to introgression from introduced *O. c. bouvieri* and *O. mykiss*. First, we examine whether all five approaches can similarly discriminate introgressed and non-introgressed populations. Second, we investigate whether three methods generating quantitative estimates of admixture provide sufficiently congruent results to allow unambiguous assignment of populations to management categories that are delineated by threshold levels of introgression. Third, we examine the influence of using alternative parental reference samples on these admixture estimates.

Methods

Tissue collection

Tissue samples (n = 23–60) were obtained from 31 populations of putative Rio Grande cutthroat trout (RGCT) in New Mexico (Table 1). These comprised 17 populations from the Rio Grande drainage, eight populations from the Pecos drainage, five populations from the Canadian drainage and a single population of unknown origin occurring in the closed Tularosa basin of southern New Mexico. Tissue samples were primarily in the form of fin-clips taken from individuals captured via electrofishing between 1996 and 2004, however for four populations (CLF, Rito Murphy, Gavilan Canyon and Yerba Creek) archived liver or muscle tissue collected at an earlier date was also included. Exact sampling pattern varied according to fish density and stream characteristics, however at all sites fish of multiple age classes were systematically sampled over a stream reach of several hundred meters or more in an attempt to minimize the effects of habitat structuring or family grouping (Hansen et al. 1997). For samples HDN, ELR and UTE, individuals were collected from two or more separate stream reaches.

In addition, tissue samples were obtained from the following: a broodstock line of *O. c. virginalis* maintained by Colorado Division of Wildlife; five domestic strains of rainbow trout which have been stocked throughout the western United States since the late 1800s; a hatchery strain of Yellowstone cutthroat trout (YSCT) originating from Yellowstone Lake and a hatchery strain of fine-spotted Snake River cutthroat trout (SRCT) which has been stocked into New Mexico waters since the 1970s (Table 1). For one of the rainbow trout samples, Steelhead we combined two sets of tissue samples collected at different times. Yellowstone and Snake River cutthroat trout are currently considered to be the same subspecies (*O. c. bouvieri*) and we found very similar allele distributions within these strains at all loci. Therefore we tested the putative Rio Grande cutthroat trout populations for their level of admixture from three different sources: Rio Grande cutthroat trout (RGCT), rainbow trout (RNBW) and Yellowstone/Snake River cutthroat trout (Y/SRCT). (Table 2)

Microsatellite analysis

DNA was extracted using the PureGene DNA Extraction Kit (Gentra Systems, Minneapolis, MN, USA) following the manufacturer's instructions. We amplified ten tetranucleotide microsatellite loci (J3, J14, K216, K222, H12, H18, H114, H118, H126, H220) all of which exhibit overlap in allele sizes between *O. c. virginalis*, *O. c. bouvieri* and *O. mykiss* (Pritchard et al. in press b). Microsatellites were amplified in 20 µl reactions and products labelled using an M13 procedure (Pritchard et al. in press b) We used the following reaction mix: 1 µl template DNA, 2 ng/ml; reverse and M13-modified forward primers, 0.2 mM each; M13-labelled oligo, 0.1 mM; dNTPs, 0.2 mM each; $MgCl_2$, 1.5 mM; Biotaq DNA polymerase (Bioline USA Inc., Canton, MA), 0.25 units; and Biotaq buffer, 1X. PCR was conducted using a MJ Research PTC-100 96 V thermocycler with the following conditions: initial denaturation 95°C (5 min), followed by 10 cycles of 94°C (30 s), 57°C (60 s), and 72°C (30 s), followed by 22 cycles of 94°C (30 s), 55°C (60 s), and 72°C (30 s), and terminating with a final extension at 72°C for 10 min. Amplification products were mixed 1:1 with 98% formamide loading dye, denatured for 3 min at 95°C, and then cooled on ice before running on 5% denaturing acrylamide gels at 35 W for 70 min. Products were detected using the ABI-377. Initial results showed multiple alleles with 1 bp, 2 bp or 3 bp size differences. Comparison of alleles between replicate genotyping runs of 15 individuals, and examination of allele size distribution over the entire dataset, suggested that the majority of observed 1–3 bp size differences were due to allele sizing errors. We therefore binned all alleles into 4 bp size categories.

Table 1 Test and reference samples used in this study

	Code	Description	*N*	Current management category	Purity assessment	Stocking record?
Test population						
Rito Angostura	ANG	RGCT Rio Grande	30	Sportfish	m, a	Y
Bitter Creek	BIT	RGCT Rio Grande	30	Core	m, a	R, Y
Cabresto Creek	CAB	RGCT Rio Grande	30	Conservation?	m	R, Y, SR
Cañones Creek	CAN	RGCT Rio Grande	30	Core	m, a, mtDNA	R, Y
Cabresto Lake Fork	CLF	RGCT Rio Grande	30	Core	m, a	R, Y
Columbine Creek	COL	RGCT Rio Grande	30	Core	m, a	R, Y
El Rito Creek	ELR	RGCT Rio Grande	40	Core	m, a	R, Y
Holden Prong	HDN	RGCT Rio Grande	23	Conservation	m, a	R, Y
Osha Creek	OSH	RGCT Rio Grande	24	Unknown	none	R?, Y?
Polvadera Creek	PVA	RGCT Rio Grande	30	Core	m, a	R, Y
Rio de las Vacas[1]	RLV	RGCT Rio Grande	30	Core	m, a	R?, Y?
Upper Comanche	UCO	RGCT Rio Grande	30	Core	m, a	R, Y
Ute Creek	UTE	RGCT Rio Grande	60	Unknown	none	R?, Y?
Cow Creek	COW	RGCT Pecos	30	Unknown	none	R, Y, SR
Dalton Creek	DAL	RGCT Pecos	30	Unknown	none	R, Y
Doctor Creek	DOC	RGCT Pecos	29	Core	m, a	R, Y
Rito los Esteros	EST	RGCT Pecos	31	Unknown	none	none
Rio Mora	MOR	RGCT Pecos	30	Core?	m	R, Y
Rio Mora Tributary	MTR	RGCT Pecos	30	Unknown	none	none
Rito del Padre	PDR	RGCT Pecos	29	Core	m, a	R, Y, SR
Rio Valdez	VDZ	RGCT Pecos	30	Core	m, a	R, Y, SR
Middle Ponil	MPO	RGCT Canadian	30	Sportfish	m, a	R, Y
Ricardo Creek	RIC	RGCT Canadian	40	Core	m, a	R, Y
Little Vermejo	VJO	RGCT Canadian	30	Core	m, a	Y
Indian Creek	IND	RGCT Tularosa	37	Core?	m	R?, Y?
Reference population						
Gavilan Canyon	US-RGCT	RGCT Rio Grande	30	Core	m, a	none
Palociento Creek	US-RGCT	RGCT Rio Grande	28	Core	m, a	none
Policarpio Creek	US-RGCT	RGCT Rio Grande	30	Core	m, a	none
Yerba Creek	US-RGCT	RGCT Rio Grande	29	Core	m, a	none
McCrystal Creek	US-RGCT	RGCT Canadian	30	Core	m, a, mtDNA	none
Rito Murphy	US-RGCT	RGCT Canadian	14	Core	m, a	none
CO Hatchery Stock	CO-RGCT	RGCT Rio Grande	30	Core	a, PINE	–
Black Canyon strain	RNBW	Rainbow hatchery	15	–	–	–
McConaughey strain	RNBW	Rainbow hatchery	29	–	–	–
Shasta strain	RNBW	Rainbow hatchery	30	–	–	–
Steelhead	RNBW	Rainbow hatchery	44	–	–	–
Tasmania strain	RNBW	Rainbow hatchery	15	–	–	–
Yellowstone cutthroat	Y/SRCT	YSCT hatchery	30	–	–	–
Snake River cutthroat	Y/SRCT	SRCT hatchery	16	–	–	–

'Current management category' indicates the current management classification of a population (New Mexico Department of Game and Fish 2002) based upon previous assessments of levels of introgression using morphometrics (m), allozymes (a), mitochondrial DNA (mtDNA) and/ or paired interspersed nuclear elements (PINEs) (Colorado Division of Wildlife, unpublished data; New Mexico Department of Game and Fish, unpublished data; Keeler-Foster 2003). 'Stocking record?' indicates whether records held by New Mexico Department of Game and Fish show trout stocking into a stream. Stocking is indicated by R (rainbow trout), Y (Yellowstone cutthroat trout, also recorded as 'black spotted trout' or 'native black spotted') or S (Snake River cutthroat trout, also recorded as 'cutthroat'). '?' indicates that the stocking record may refer to more than one stream with the same name

[1] The population in Rio de las Vacas was transplanted from Rio Puerco: stocking records refer to Rio Puerco

Choice of reference populations

The a-priori identification of pure populations of Rio Grande cutthroat trout is problematic. Stocking of non-native *Oncorhynchus* taxa has been intensive and widespread within the subspecies' range, even in remote wilderness areas, and migration of stocked fish between streams is to be expected. We used a combination of approaches to select suitable Rio Grande cutthroat trout reference samples for this study. First, we used historical stocking records, maintained by New Mexico Department of Game and Fish since the late 1800s, to identify populations on public land that were not recorded as receiving transplants of non-native

Table 2 Observed and unbiased expected heterozygosity and F_{is} over all loci for each population sample

	H_e	H_o	F_{is}
Test population			
Rito Angostura	0.56	0.72	–0.29*
Bitter Creek	0.47	0.46	0.03*
Cabresto Creek	0.70	0.66	0.05
Cañones Creek	0.15	0.11	0.29*
Cabresto Lake Fork	0.57	0.53	0.06
Columbine Creek	0.51	0.46	0.10
El Rito Creek	0.56	0.59	–0.05*
Holden Prong	0.80	0.74	0.08*
Osha Creek	0.19	0.20	–0.05
Polvadera Creek	0.05	0.05	0.05
Rio de las Vacas	0.62	0.62	–0.01
Upper Comanche	0.56	0.52	0.07*
Ute Creek	0.53	0.49	0.09*
Cow Creek	0.65	0.67	–0.02*
Dalton Creek	0.26	0.24	0.10
Doctor Creek	0.45	0.49	–0.09
Rio Mora	0.22	0.20	0.06
Rio Mora Tributary	0.32	0.32	0.00
Rito los Esteros	0.43	0.35	0.18*
Rito del Padre	0.51	0.50	0.01
Rio Valdez	0.55	0.67	–0.21
Middle Ponil	0.75	0.71	0.05*
Ricardo Creek	0.60	0.58	0.03
Little Vermejo	0.54	0.56	–0.02
Indian Creek	0.58	0.57	0.02
Reference population			
Gavilan Canyon	0.53	0.48	0.09*
Palociento Creek	0.45	0.44	0.02
Policarpio Creek	0.42	0.45	-0.07
Yerba Creek	0.49	0.43	0.12*
McCrystal Creek	0.30	0.34	-0.12
Rito Murphy	0.31	0.31	0.00
CO Hatchery Stock	0.67	0.66	0.01
Black Canyon strain	0.65	0.51	0.23*
McConaughey strain	0.64	0.57	0.12*
Shasta strain	0.59	0.63	-0.06
Steelhead	0.75	0.63	0.17*
Tasmania strain	0.62	0.57	0.08
Snake River	0.71	0.70	0.02
Yellowstone	0.78	0.77	0.00

Samples deviating significantly from Hardy–Weinberg equilibrium ($L < 0.05$) are indicated by *

Oncorhynchus taxa. Although levels of introgression from *O. c. bouvieri* had not been quantified for any population, six populations within this subset had previously been estimated to have zero introgression from *O. mykiss*, based on allozyme data (New Mexico Department of Game and Fish, unpublished data). We combined these six populations to form our first reference sample, hereafter referred to as 'unstocked RGCT' (US-RGCT). The sample included populations from the Rio Grande and Canadian drainage, however no suitable population was identified from the Pecos drainage. As an alternative reference sample, we utilized a broodstock line of Rio Grande cutthroat trout maintained by Colorado Division of Wildlife, hereafter referred to as 'CO RGCT'. This line contains genetic material from several *O. c. virginalis* populations within the Rio Grande drainage in Colorado and has previously been confirmed as containing < 0.1% non-native genetic material using PINEs (Colorado Division of Wildlife unpublished data).

Population genetic statistics

Observed and unbiased expected heterozygosity (Nei 1978) and inbreeding coefficients (F_{is}, Weir and Cockerham 1984) were calculated for all loci in each population sample using Genetix 4.02 (Belkhir et al. 2001). Tests for conformation to Hardy Weinberg equilibrium were performed for each locus in each population using an exact test implemented in Genepop 3.4 (Raymond and Rousset 1995). We also used an exact test implemented in Genepop to test for linkage equilibrium over all locus pairs in each population. Significance of Hardy–Weinberg tests over all loci in each population, and significance of linkage equilibrium tests over all locus pairs in each population, and over all populations for each locus pair, was assessed by calculating the likelihood of obtaining the observed number of significant tests by chance alone. To do this we used the binomial likelihood function (Chapman et al. 1999; Kinnison et al. 2002). The null hypothesis, for example no deviation from Hardy–Weinberg equilibrium or linkage equilibrium, was rejected where $L < 0.05$.

It can be difficult to ascertain the cause of statistically significant linkage disequilibrium or heterozygote deficiency within a microsatellite dataset. Significant linkage disequilibria may arise as a result of the recent mixing of genetically distinct lineages (Nei and Li 1973) or a low effective population size (Bartley et al 1992), even where loci are on different chromosomes. Similarly, although heterozygote deficiencies may indicate the presence of null alleles or other genotyping artefacts, they may also reflect actual population genetic processes. Populations of trout isolated in small streams may frequently deviate from Hardy-Weinberg equilibrium, both because these populations may contain a relatively few families (Hansen and Jensen 2005) and because fine-scale population sub-structuring may be present (Wofford et al. 2005). Both linkage disequilibria and deviations from Hardy-Weinberg equilibrium can also be a by-product of the sampling scheme used when collecting tissue for analysis. In an attempt to identify whether heterozygote deficiencies in our dataset were caused by genotyping artefacts, we

first assumed that all sampled populations were, in reality, in Hardy–Weinberg equilibrium and used the program Micro-checker (van Oosterhout et al. 2004) to identify sample-locus combinations where null alleles might be present, based on the observed excess of homozygotes. We then examined these sample-locus combinations for the presence of non-amplifying individuals, which would imply the existence of null homozygotes. Where Micro-checker suggested a locus in a population to contain null alleles, and non-amplifying individuals were observed, we concluded that one or more null alleles were truly present.

Correspondence analysis

We made a preliminary exploration of the data using factorial correspondence analysis (FCA), adapted for use with diploid genetic data following She et al. (1987), and implemented in Genetix. FCA is an exploratory technique, suitable for categorical data, which allows investigation of correspondence between rows (e.g., individuals) and columns (e.g., alleles) in a two-way table. It enables visualization of individuals in multidimensional space, with no a-priori assumptions about grouping, using each allele as an independent variable. Axes are generated from combinations of alleles that explain portions of the total observed 'inertia' of the table, defined as the Pearson χ^2 statistic for the table divided by the overall number of observations. Hence those alleles which exhibit the strongest non-random association with groups of individuals will contribute most to the axes. Missing data at one or more loci can create bias in the FCA projection (Roques et al. 2001), however in this study exclusion of individuals with incomplete multilocus genotypes did not substantially change the results, and we therefore included all 1,164 individuals in the analysis.

Maximum-likelihood assignment tests

Several authors have used maximum-likelihood based genetic classification methods to investigate introgression within populations, by examining the likelihood with which individuals are assigned to a non-native reference sample (Wares et al. 2004; Taylor et al. 2007). We implemented a Bayesian approach (Rannala and Mountain 1997) available in Geneclass 1.0.02 (Cornuet et al. 1999), first to assign individuals to their most likely population of origin based on observed microsatellite allele frequencies, and second to assess the probability that an individual belonged to each alternative population, by comparing its genotype to that of 10,000 simulated genotypes generated by randomly taking alleles according to their estimated frequencies in that population. The method assumes Hardy–Weinberg equilibrium within populations. We initially provided Geneclass with a reference dataset containing microsatellite data for Yellowstone cutthroat trout, Snake River cutthroat trout, all rainbow trout samples, and either 'CO RGCT' or 'unstocked RGCT', and forced the program to assign each test individual to one of the 8 or 13 reference samples. Subsequently, we provided the program with the entire microsatellite dataset, and enabled it to assign an individual to any one of the 39 possible population samples, implementing the 'leave one out' option, which causes the individual of interest to be removed from the data set prior to calculation of sample allele frequencies. We considered an individual to contain non-native genetic material when its estimated probability of belonging to one of the non-native reference samples was > 0.01.

Allele frequency approach

The genetic contribution of two or more parental populations to an admixed population can be estimated simply from the observed allele frequencies in those populations using a least-squares method (*sensu* Chakrabory 1986) implemented in the program Admix 2.0 (Dupanloup and Bertorelle 2001). This approach assumes that allelic frequencies estimated from the reference and test samples are identical to those in the true parental and hybrid populations immediately after the admixture event, which occurred at a single point in time. We provided the program with an input file containing allele frequencies for a test population and three putative 'parental populations': the pooled rainbow trout samples, the pooled Yellowstone and Snake River cutthroat trout samples, and a sample of Rio Grande cutthroat trout. We chose to pool samples to create our parental groups, following Dupanloup et al. (2004), as a pooled sample is more likely to contain at least some of the alleles present in the true parental population. In order to investigate the influence of using different Rio Grande cutthroat trout reference samples on the estimation of admixture levels, we ran the analysis twice for each population, once using 'unstocked RGCT' as a parental reference sample and once using 'CO RGCT' as a parental reference sample. We calculated 95% confidence limits of admixture proportions from 2,000 bootstrap replicates.

Coalescent approach

Bertorelle and Excoffier (1998) and Dupanloup and Bertorelle (2001) have developed a method, based on coalescent theory to estimate admixture proportions (mY) in a population utilizing information about both allele frequencies and the mutational distance between alleles in the hybrid and parental population. This method is also implemented in Admix 2.0. It is expected to perform better than the simple frequency based estimator where parental taxa have been isolated for a sufficient time for mutational changes to contribute to inter-taxonomic allele size variation, as is expected to have been the case with *O. mykiss*, *O. c. bouvieri* and *O. c. virginalis* (Bertorelle and Excoffier 1998). The method assumes, amongst other things, that the reference samples are taken from the true parental populations and that the admixture event occurred at a single point in time. We provided the program with input files as described previously and ran the analysis with the two alternative RGCT reference samples. As recommended by Bertorelle and Excoffier (1998), we used the squared difference in tetranucleotide repeat number as an estimation of coalescence time, assuming a strict stepwise model of microsatellite mutation. As the admixture event is expected to be relatively recent, we assumed no subsequent mutational changes in alleles. Again, we calculated confidence limits from 2,000 bootstrap replicates.

Bayesian estimation of admixture proportions

Structure (Pritchard et al. 2000) is a model-based Bayesian, Markov chain Monte Carlo (MCMC) approach that uses the assumption of Hardy–Weinberg equilibrium and linkage equilibrium within populations to estimate, amongst other things, the number of discrete populations within a sample and the admixture coefficient (q) of each individual within a sample. The program can be used to estimate admixture coefficients where no pure native reference population is available (Beaumont et al. 2001; Hansen et al. 2001). We initially used Structure to confirm that there were 7 distinct populations represented by our Yellowstone cutthroat trout, Snake River cutthroat trout and rainbow trout reference samples, and 6 distinct populations represented by our 'unstocked RGCT' reference sample. This was done by pooling all reference samples and comparing the likelihood of the data assuming that they originated from 1 to 20 populations. We then used Structure to estimate levels of admixture in each test population. For each run of Structure we provided the program with microsatellite data for all rainbow trout, Yellowstone cutthroat trout and Snake River cutthroat trout reference samples and the RGCT test sample of interest. The model was forced to consider each of the reference populations as 'pure' (i.e., non-admixed individuals), while individuals within the test sample were allowed to have an admixed ancestry. Allele frequencies were assumed to be uncorrelated between populations. We used a burn-in period of 50,000 steps and then proceeded with 100,000 MCMC replicates. Each analysis for each population was repeated three times to ensure consistency of results. In cases where the three replicate MCMC runs were found to converge on different solutions, we ran more replicates and selected the solution with the highest log likelihood.

First, we ran Structure for each test population using no RGCT reference sample. The program was informed that there were 8 populations in the data set. For each individual in the test sample, the proportion of its ancestry derived from population 8 (q8) was assumed to approximate the Rio Grande cutthroat trout admixture coefficient for that individual. Second, we provided Structure with microsatellite data for the 'CO RGCT' reference sample. The program was again informed that there were 8 populations in the data set, hence in this case the program was forced to assign each RGCT test individual to one or more of the reference populations. Again, q8 (proportion of ancestry derived from CO RGCT) was assumed to approximate the RGCT admixture coefficient for each individual. Third, we provided Structure with microsatellite data for the 'unstocked RGCT' reference sample. The program was informed that there were 13 populations in the data set, and the RGCT admixture coefficient for each test individual was assumed to approximate the sum of q8–q13 (proportion of ancestry derived from each of the 6 RGCT reference populations, hereafter referred to as '*sum q8–q13*').

The mean individual q8 or *sum q8–q13* in each of the 25 Rio Grande cutthroat trout test samples was used as an estimate of population-level admixture. We estimated 95% confidence levels of this population-level admixture following Hansen et al. (2001). In order to account for variation among individuals and variation in q8 or *sum q8–q13* values in different MCMC replicates we sampled individual q8 or *sum q8–q13* values from 20 single steps of the Markov chain (that is, a burn-in period of 50,000 steps followed by just one MCMC replicate). We drew 2,000 bootstrap samples of size *n* from these 20*n* individual q8 or *sum q8–q13* values and estimated confidence intervals from the distribution of mean q8 or *sum q8–q13* values. Where replicate runs converged on different solutions,

we performed additional single step MCMC runs and sampled only from those that converged on the solution which we had previously determined to have the maximum log likelihood.

Results

Population genetic statistics

Observed and expected heterozygosities over all loci for each sample, and significance of deviations from Hardy–Weinberg equilibrium are shown in Table 1. Results for each locus-sample combination are provided in Appendix 1. Ten of the 25 samples from test populations and 4 of the 14 samples from reference populations deviated significantly from Hardy–Weinberg equilibrium. The majority of these deviations were due to significant heterozygote deficiencies at multiple loci (CAN, EST, HDN, UCO, Yerba Creek, Steelhead) or significant heterozygote deficiencies at some loci combined with significant heterozygote excess at other loci (BIT, COW, ELR, MPO, Gavilan Canyon, McConaughey), however sample ANG exhibited overall heterozygote excess. Some heterozygote deficiencies in Gavilan Canyon, Yerba Creek and Steelhead may have arisen because these samples combined tissues collected in different years. Heterozygote excess in ANG does not appear to be due to the presence of F1 hybrids within the sample. Assuming that all samples were, in reality, in Hardy–Weinberg equilibrium, Micro-checker suggested the presence of null alleles for 31 locus-sample combinations (J3: 3 samples; J14: 1 sample; K216: 6 samples; K222: 5 samples; H12: 3 samples; H18: 7 samples; H114: 2 samples; H118: 1 sample; H126: 3 samples; H220: no samples). We observed non-amplifying individuals in nine of these locus-sample combinations (J3: none; J14: Black Canyon; K216: McConaughey, Steelhead; K222: McConaughey, Steelhead; H18: McConaughey, Steelhead; H114: none; H118: Black Canyon; H126: COL). These results suggest that null alleles may be present at loci K216, K222 and H18 within *O. mykiss* and at locus H126 within at least one sample of *O. c. virginalis*. Locus K216 amplifies poorly in most *O. mykiss* samples, and we have previously noted evidence for null alleles at H126 within other population samples of *O. c. virginalis*, *O. c. pleuriticus* and *O. c. stomias* (Pritchard et al. in press b).

We observed significant overall linkage disequilibrium within nine samples (CAB: 23 significant deviations from linkage equilibrium observed within 45 possible pairs of polymorphic loci, $L < 0.0001$; COW: 19 significant deviations within 45 pairs, $L < 0.0001$; MPO: 20 significant deviations within 45 pairs, $L < 0.0001$; McConaughey: 12 significant deviations within 43 pairs, $L < 0.0001$; Shasta: 11 significant deviations within 45 pairs, $L < 0.0001$; EST: 10 significant deviations within 45 pairs, $L = 0.0003$; MTR: 7 significant deviations within 28 pairs, $L = 0.001$; COL: 8 significant deviations within 45 pairs, $L = 0.008$; Yerba Creek: 7 significant deviations within 45 pairs, $L < 0.038$). We have previously noted evidence for fine-scale population genetic structuring within remnant *O. c. virginalis* populations (Pritchard et al. in press a), and we suspect that this phenomenon, rather than recent hybridization, may be responsible for the significant deviations from linkage equilibrium observed in samples COL, EST, MTR and Yerba Creek. In contrast, results from subsequent admixture analyses suggested that the high levels of linkage disequilibrium observed in CAB, COW and MPO were due to recent hybridization events. Similarly, although we did not know the recent history of the McConaughey and Shasta *O. mykiss* strains, the significant linkage disequilibrium observed within these samples suggested that they might contain two or more rainbow trout lines that had recently been mixed. We therefore omitted these latter five samples when assessing the significance of linkage disequilibrium for each locus pair over all populations. Following omission of these samples, 2 locus pairs exhibited overall significant deviation from linkage equilibrium, (J3 and K216: 5 significant linkage disequilibria within 25 samples, $L = 0.03$; H12 and H18: 6 significant linkage disequilibria within 32 samples, $L = 0.02$). These deviations were no longer significant when COL, EST, MTR and Yerba Creek were also removed.

To test whether physical linkage between J3 and K216, or H12 and H18, or the presence of null alleles at K216, K222, H18 and H126, were biasing our results, we repeated all analyses omitting the latter four loci. We did not omit loci J14 and H118 as strong evidence for null alleles at these loci was restricted to the small Black Canyon *O. mykiss* sample and may reflect specific conditions when this sample was genotyped.

Correspondence analysis

Figure 1 illustrates the results of the factorial correspondence analysis. Although the analysis was performed on the entire dataset, populations are plotted on four different scattergraphs for ease of interpretation. Alleles at all loci contributed to each of the two axes shown. Rio Grande cutthroat trout and rainbow trout separated along Axis 1 (4.2% of total inertia),

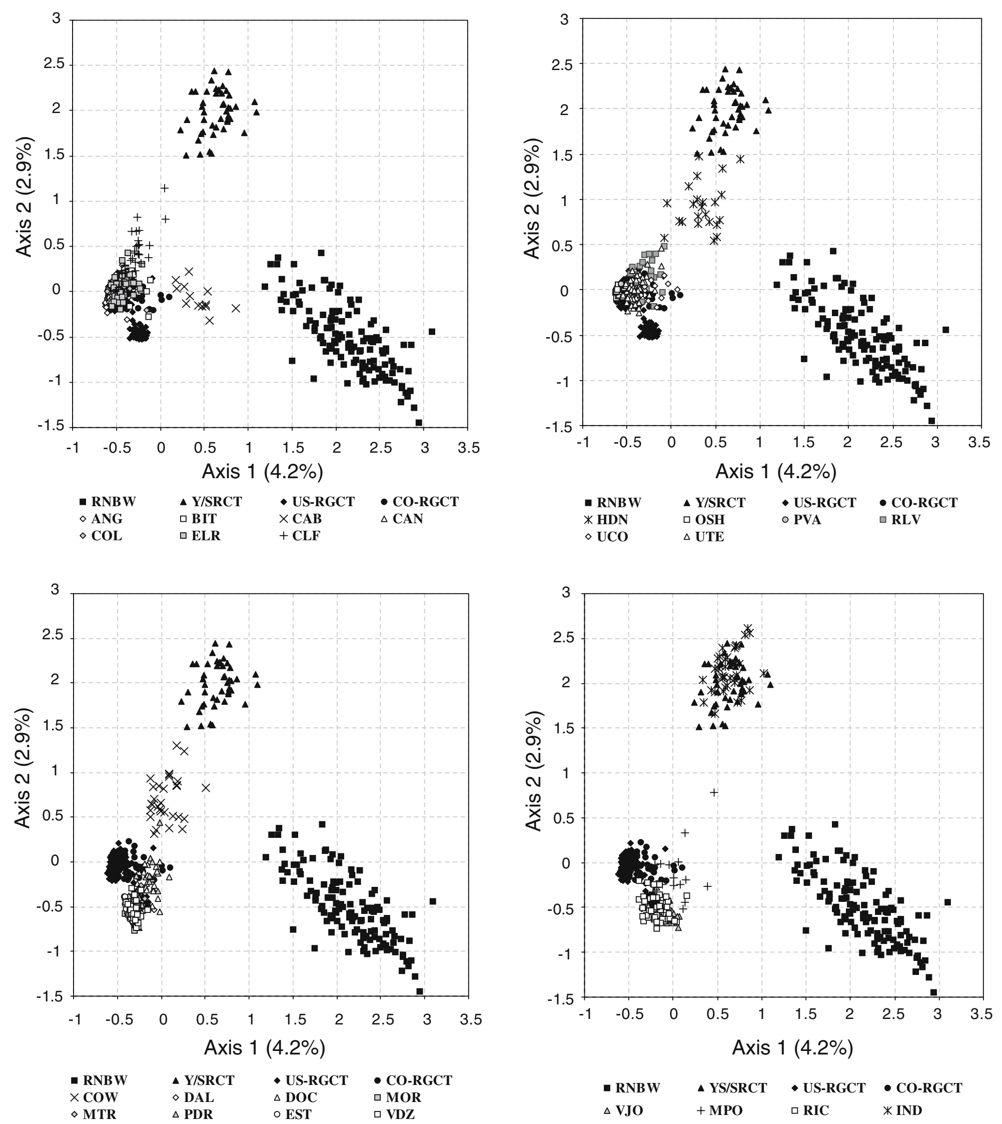

Fig. 1 Scattergraphs showing position of individuals along Axes 1 and 2 generated by the Factorial Correspondence Analysis. Population codes are defined in Table 1

and RGCT and Y/SRCT separated along Axis 2 (2.9% of total inertia). Whilst the majority of individuals from the RGCT test samples clustered together closely on the scattergraph, the position of some individuals clearly suggested a non-native genetic influence. All individuals from IND clustered with individuals from the Y/SRCT reference sample. Individuals from CLF, COW, HDN and MPO were located midway between the Y/SRCT and RGCT clusters, demonstrating that these populations contain some alleles characteristic of RGCT and some alleles more characteristic of Y/SRCT. Similarly, individuals from CAB clustered between RGCT and rainbow trout, demonstrating the presence of alleles characteristic of rainbow trout within this population. The position of a small number of individuals from DOC, ELR, RIC, RLV and PDR also suggested some possible non-native introgression within these populations.

The relative position of most individuals on the scattergraph was not greatly changed when loci K216, K222, H18 and H126 were removed from the analysis, however, results were more strongly suggestive of non-native introgression in PDR, RLV and a single individual from RIC, and also suggested the presence of non-native genetic material within some individuals from BIT, UCO and UTE.

Maximum-likelihood assignment tests

When CO-RGCT was included as the *O. c. virginalis* reference, 653 (80%) of test individuals were not assigned to any reference sample with $P > 0.01$. Of those that could be assigned to a sample, all were assigned to CO-RGCT, with the exception of 26 individuals from IND, which were assigned to Yellowstone cutthroat trout. No individual was assigned to any rainbow trout

sample with $P > 0.01$. Results were almost identical when unstocked RGCT was included as the *O. c. virginalis* reference sample: 647 individuals were not assigned to any reference sample; 143 individuals were assigned to one of the US-RGCT samples; 26 individuals from IND were assigned to YSCT and no individuals were assigned to rainbow trout. When we provided Geneclass with the entire dataset, and allowed individuals to originate from any of the 39 alternative population samples, the majority of individuals were assigned with the highest probability to their population of origin. Again, all RGCT test individuals were rejected from any rainbow trout sample ($P < 0.01$), however, 25 individuals from IND were not rejected from the Yellowstone cutthroat trout sample. No individual from IND was assigned to any other RGCT sample with $P > 0.01$ in any analysis.

Results of assignment tests were only slightly changed when loci K216, K222, H18 and H126 were removed from the analyses, and differences could be attributed to the reduced number of loci rather than specifically to the exclusion of null alleles or linked markers. Where Geneclass was forced to assign individuals to reference samples, with either the US-RGCT or CO-RGCT sample included, 30 or 32 individuals from IND, 3 individuals from COW and 3 individuals from HDN were assigned to Yellowstone cutthroat trout. Similarly, when we allowed individuals to be assigned to any population, 32 individuals from Indian Creek and 3 individuals each from Cow Creek and Holden Prong were not rejected from YSCT and/or SRCT.

Allele frequency approach

Figure 2a shows RGCT admixture coefficients estimated using the allele frequency approach implemented in Admix 2.0. Dotted lines indicate RGCT admixture coefficients of 0.99 and 0.90, assumed to correspond to the threshold levels of 1% and 10% non-native introgression used to assign cutthroat trout populations to different genetic management categories (Anonymous 2000). Estimated levels of RNBW and Y/SRCT admixture are shown in Figures 2b and c, with dotted lines indicating admixture coefficients of 0.01 and 0.1. The allele frequency approach estimated a RGCT admixture coefficient > 0.9 for only four of the 25 test populations, and for some populations estimates varied substantially with the RGCT reference sample used. All populations within the Canadian and Pecos drainages were estimated to contain 30–70% non-native genetic material, which was primarily attributed to rainbow trout. Samples COW, CLF, HDN and IND were estimated to contain a substantial amount of genetic material originating from Y/SRCT. In some cases, estimated RNBW admixture coefficients were negative.

Removal of K216, K222, H18 and H126 from the data set did not have a consistent effect on the results: estimated RGCT admixture coefficient increased or decreased, in most cases by a relatively small amount, depending upon the test sample and RGCT reference sample provided. In all but three cases (COL and ELR with the US-RGCT reference sample; RLV with the CO-RGCT reference sample), this change was not sufficient to cause a population to be assigned into a different 'management category' as defined by the Position Paper.

Coalescent approach

Figures 3a, b and c show the levels of RGCT, RNBW and Y/SRCT admixture estimated for each population using the coalescent approach implemented in Admix 2.0. Again, dotted lines indicate non-native RGCT admixture coefficients of 0.99 and 0.9, or non-native admixture coefficients of 0.01 and 0.1. Results differed substantially from those obtained using allele frequencies alone. Estimated RGCT admixture coefficients were high for the majority of test populations, with only eight: CAB, CLF, COW, DOC, HDN, IND, MPO and RLV, estimated to have a RGCT admixture coefficient < 0.9. Sample IND exhibited a very low estimated RGCT admixture coefficient in combination with a high Y/SRCT admixture coefficient. Hence, results were qualitatively similar to those obtained using correspondence analysis, even thought the latter approach does not take into account information about allele size. Generally similar results were obtained when using the two alternative reference samples, although RGCT admixture coefficient estimated using the CO-RGCT reference sample was often smaller than that estimated using the US-RGCT reference sample. Again, Admix frequently estimated negative RNBW or Y/SRCT admixture coefficients for test populations. Populations exhibiting a RGCT admixture coefficient > 0.99, therefore, could also exhibit a RNBW or Y/SRCT admixture coefficient > 0.01 as a result of a negative admixture coefficient being estimated for the third putative parental population. For the same reason, estimated RGCT admixture coefficients for several populations were > 1.

Removal of K216, K222, H18 and H126 from the analyses resulted in a decreased, and frequently negative, estimated RNBW admixture coefficient for most samples. This was accompanied with an increase in the estimated RGCT and YSCT admixture coefficients.

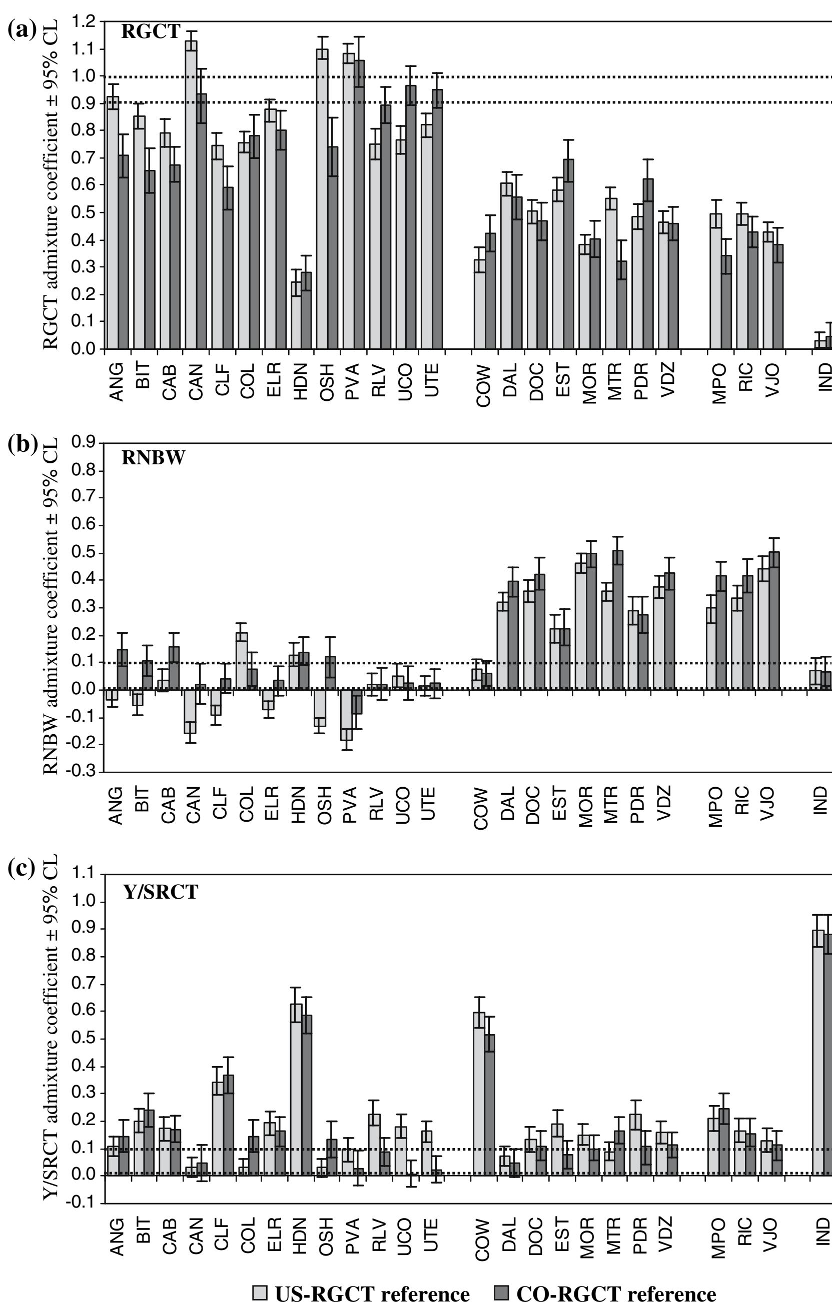

Fig. 2 Admixture coefficients as calculated using the allele frequency approach implemented Admix 2.0. (**a**) RGCT admixture coefficient; (**b**) RNBW admixture coefficient; (**c**) Y/SRCT admixture coefficient

Irrespective of the RGCT reference sample provided, samples CAB, CLF, HDN, COW, MPO and IND continued to exhibit a RGCT admixture coefficient > 0.9; samples DOC and RLV exhibited a RGCT admixture coefficient < 0.99 and all other samples exhibited a RGCT admixture coefficient > 1.

Structure

When we used Structure to estimate the number of populations present in our pooled reference sample, log likelihood of the solution increased from $K = 1$ until it reached a plateau at $K = 14$. However, the value of ΔK (Evanno et al. 2005) was maximized at $K = 4$, with the four identified groups comprising Y/SRCT, RNBW, Rio Grande RGCT and Canadian River RGCT. We chose to define $K = 14$ as we know a-priori that the majority of our reference samples are taken from demographically independent populations. However, for many test populations, we have previously run the same analyses defining the reference samples to comprise just three groups: Y/SRCT,

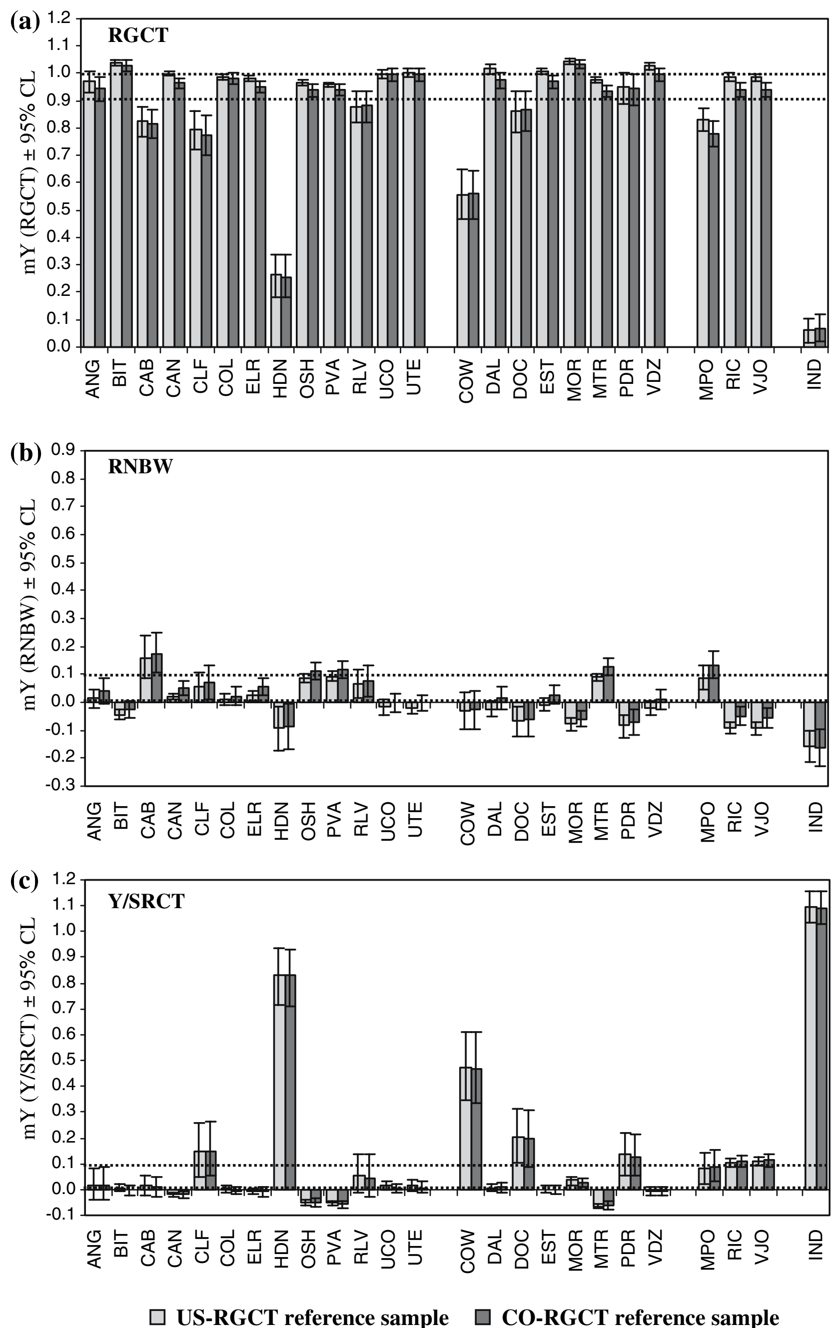

Fig. 3 Admixture coefficients as calculated using the coalescent approach implemented Admix 2.0. (**a**) RGCT admixture coefficient; (**b**) RNBW admixture coefficient; (**c**) Y/SRCT admixture coefficient

RNBW and RGCT, and found very similar results to those presented here (V. L. Pritchard, unpublished data).

Figure 4 shows mean population-level RGCT admixture coefficient (q8 or *sum q8–q13*) for all test populations as estimated using Structure. In all cases, mean q8 or *sum q8–q13* was less than one. This is because Structure estimated a non-zero, but often negligible, contribution of each of the reference populations to each test individual irrespective of its genotype. Results clearly varied according to the reference sample used. Where no reference sample was provided, all replicate runs converged on the same solution, and Structure estimated relatively high RGCT admixture coefficients for all populations. However those populations that exhibited the lowest values of q8 were generally also those that appeared most introgressed using the alternative approaches. Where the 'CO RGCT' reference sample, derived from the Rio Grande drainage, was used, estimated RGCT admixture coefficient varied greatly between test populations. All test populations within the Rio Grande

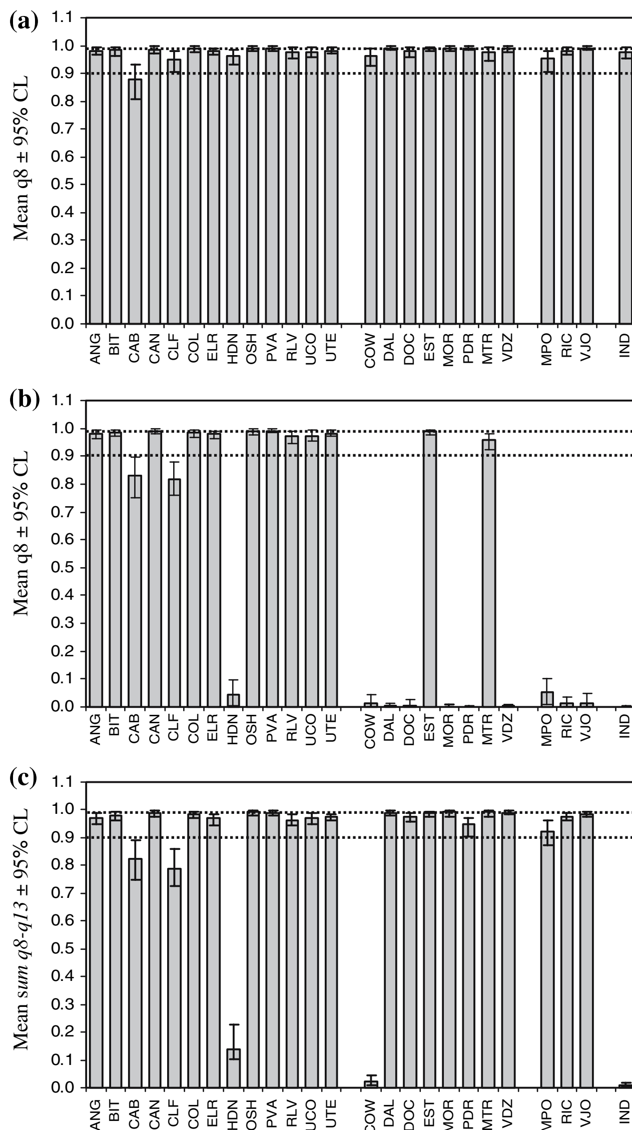

Fig. 4 Mean q8 or mean *sum q8-q13*, calculated by Structure and assumed to approximate RGCT admixture coefficient. (**a**) No reference sample; (**b**) 'CO RGCT' used as reference sample; (**c**) 'Unstocked RGCT' used as reference sample

drainage exhibited a high mean q8, which was constant over Structure runs: that is, individuals in the population were estimated to derive most of their ancestry from 'CO RGCT'. In contrast, for most populations within the Canadian and Pecos drainages, replicate Structure runs converged on different solutions, and the solution with the highest log likelihood generally estimated a low mean q8. Hence, most individuals in these population samples were estimated to derive most of their ancestry from a reference sample other than 'CO RGCT'. When using the 'unstocked RGCT' reference sample, which contained individuals from both the Rio Grande and the Canadian drainages, replicate structure runs generally converged on the same solution, and estimated population-level RGCT admixture coefficients (*sum q8–q13*) were relatively high for all test populations except Indian Creek, Holden Prong and Cow Creek. Again, a number of populations that appeared to be more introgressed in other analysis also exhibited lower estimated RGCT admixture coefficients using Structure.

RGCT admixture coefficients estimated using Structure from the dataset omitting K216, K222, H18 and H126 were generally slightly smaller than those estimated from the full dataset, irrespective of the reference sample provided. The difference between the two estimates was always less than 0.05. Exceptions to this pattern were CLF, HDN and COW, which exhibited a slightly higher RGCT admixture coefficient estimated from the reduced dataset when the 'CO-RGCT' or 'US-RGCT' reference sample was provided, and RIC and VDZ, for which a large RGCT admixture coefficient became the marginally more likely solution when using the 'CO RGCT' reference sample.

Discussion

Utility of alternative approaches for investigation of admixture in *O. c. virginalis*

In this paper we have used five different approaches, applied to data from 10 microsatellite loci, to investigate non-native admixture within a taxon of conservation concern. All approaches reveal one population that appears to consist entirely of non-native individuals, and three out of the five approaches identify five populations that appear to contain substantial amounts of non-native introgression. However quantitative estimates of admixture for each population vary both with the method used and the reference sample provided. Thus, results do not clearly discriminate between pure populations and those with low levels of non-native admixture, and hence do not enable unambiguous assignment of populations into management categories defined by threshold levels of introgression.

Factorial correspondence analysis was developed as a method to visually explore the variance in a data set and cannot be used to provide a quantitative estimate of admixture levels. Nevertheless, comparison of the position of individuals in the FCA scattergraphs with admixture coefficients estimated using Structure or the

coalescent approach in Admix 2.0 shows that clearly introgressed individuals and populations in the data set can be readily identified visually. Hence FCA is a valuable first step in identifying hybridized populations from a set of non-diagnostic microsatellite data. This approach has the additional advantage of not requiring pure reference samples to be pre-defined.

Assignment tests, as implemented by Geneclass, are clearly not suitable for discriminating introgressed from non-introgressed populations using our dataset. Several *O. c. virginalis* populations appeared, from the results of other analyses, to contain substantial amounts of non-native genetic material, however no individual from these populations was assigned to a non-native reference sample with $P > 0.01$. Although assignment tests may perform better where the hybridizing taxa are more closely related and hence less divergent in allelic composition, we suggest that results of such tests should not be used to make inferences about the purity of a population in the absence of additional supporting analyses. We note that individuals from populations which we believe to be introgressed were frequently not rejected from other introgressed samples, hence the utility of the assignment test may be improved by including a known hybrid population as a reference sample.

Estimates of RGCT admixture based on allele frequencies alone implied the presence of non-native introgression in most populations tested, and non-native genetic contributions of more than 50% to all populations in the Canadian and Pecos drainages. We consider that these results are unlikely to reflect reality for several reasons. First the allele frequencies in the reference samples that we provided are expected to differ substantially from those that occurred in the true parental populations at the time of the admixture event. Second, we would expect a population containing 50% genetic material from rainbow trout to exhibit evidence of its hybrid origin in the morphological appearance of its constituent individuals. With the exception of MPO, and COW, which was previously suspected on the basis of spotting pattern to contain some non-native genetic material, the superficial morphology of fish in the Canadian and Pecos test populations conforms to that expected for pure *O. c. virginalis* (Y. Paroz, personal communication).

Both approaches implemented in Admix frequently resulted in estimated admixture coefficients less than 0 or greater than 1. This has been frequently observed in other studies (Bertorelle and Excoffier 1998; Vernesi et al. 2003; Choisy et al. 2004; Dupanloup et al. 2004; Fraser and Bernatchez 2005), and is expected to occur when the admixture model assumed by Admix is violated, for example, when the allele frequencies in a putative parental samples differ from those in the true parental population, or when more than one admixture event took place. A negative admixture coefficient for a putative parental population indicates that it did not plausibly contribute to the test population under consideration. In a situation where populations are to be placed in management categories according to their level of non-native introgression, this phenomenon further complicates the interpretation of results as, when more that two parental taxa are involved, a population with a native admixture coefficient of 1 can also have a non-native admixture coefficient > 0. A management agency might chose to estimate the level of introgression in a population by taking only the native admixture coefficient into account, or by summing all positive non-native admixture coefficients, alternative approaches that could lead to the same population being placed in different management categories, and hence subject to very different management activities, on the basis of the same results. For example, taking the former approach, and basing our categories on admixture coefficients estimated using the CO-RGCT reference sample, 18 of the 25 *O. c. virginalis* populations in this study conform to the 'Conservation' category; taking the latter approach, only 12 populations conform to this category.

In contrast to the results generated using Admix 2.0, estimates of RGCT admixture coefficient using Structure were strongly influenced by the use of different RGCT reference samples. Where no RGCT reference sample was used, a 'RGCT admixture coefficient' (mean q8) >0.9 was estimated for every population except Cabresto Creek. Notably, Indian Creek individuals were estimated to derive most of their ancestry from population 8 rather than from the Yellowstone cutthroat trout or Snake River cutthroat trout reference sample. This is not surprising: even though the Indian Creek fish, on the basis of all other analyses, appear to be introduced *O. c. bouvieri*, they are clearly a distinct population from the Y/SRCT reference populations, and Structure recognizes them as such. Such a result demonstrates that mean q8 cannot necessarily be assumed to approximate 'RGCT admixture coefficient' when no RGCT reference sample is provided. Where Structure was provided with a RGCT reference sample containing only genetic material from the Rio Grande drainage, the majority of individuals from Pecos and Canadian populations were estimated, with the highest likelihood, to derive most of their ancestry from Y/SRCT or rainbow trout. In contrast, when the reference sample contained genetic material from both the Rio Grande and Canadian drainages,

admixture coefficients estimated using Structure most closely corresponded with those estimated using Admix. Such variation in results when using two different RGCT reference samples emphasizes the importance of taking into account possible genetic differences between populations and drainages when choosing putative parental reference populations for the estimation of admixture coefficient. *Oncorhynchus clarkii virginalis* populations in the Rio Grande drainage are known to be moderately genetically differentiated from those in the Pecos and Canadian drainages, as can be noted from the presence of two separate RGCT clusters in the FCA scattergraph (Fig. 1, Pritchard and Cowley 2006). The low estimates of RGCT admixture in populations from the Pecos and Canadian drainages when using a reference sample derived from the Rio Grande drainage alone are clearly an artifactual result of the limited range of genetic variation present in this sample. The poor reliability of results when using this reference sample could be inferred from the observation that replicate runs of the MCMC frequently converged on different solutions.

We chose to investigate admixture in *O. c. virginalis* using five readily accessible approaches that had frequently been applied in published studies on other taxa. There are several alternative methods for estimating admixture proportions from microsatellite data, applicable to situations where more than two parental populations have contributed to the admixed population, that we have not explored in this paper. Wang (2003) presents a maximum likelihood approach, implemented in the program Leadmix, which estimates the contribution of two or more parental populations to an admixed population, taking into account genetic drift in the parental and admixed populations since the hybridization event. This approach is highly applicable to the situation considered here, and has been used to investigate admixture involving two parental groups in several taxa (Larsen et al. 2005; Belle et al. 2006; DeKoning et al. 2006; Hansen et al. 2006). However we found Leadmix to be extremely computationally intensive: for most populations tested we terminated the program after it had been running on a fast personal PC for several weeks, but before it had reached a solution. We did not, however, exhaustively investigate the reason for these long run times. Excoffier et al. (2005) have recently introduced an approach based on Approximate Bayesian Computation which performs similarly to Wang's approach where the admixture event is recent, and which we have not tested using our data. The program BAPS 4.13 (Corander and Marttinen 2006) implements a Bayesian approach to fulfil many of the functions available in Structure, but with a more rapidly performing algorithm. Although estimating admixture levels using additional independent approaches may improve our discrimination of relative levels of introgression in different populations, we have no a-priori reason to assume any quantitative admixture estimate is less biased than any other, and hence we anticipate that ambiguity in assigning populations to different genetic management categories will remain.

Genetic status of *O. c. virginalis* populations

Although we are unable to unambiguously assign all *O. c. virginalis* populations included in this study to a specific genetic management category, as defined by the Position Paper (Anonymous 2000), our results do suggest that some populations are currently mis-categorized. For example, the putative 'Core *O. c. virginalis*' population in Indian Creek actually appears to consist entirely of introduced *O. c. bouvieri*. This population is located in a closed basin and was previously believed to have originated from a transplant of Rio Grande cutthroat trout from the adjacent Sacramento Mountains, where the subspecies no longer occurs (Behnke 1992). The population was utilized as a source for an abandoned *O. c. virginalis* broodstock project in the 1980s (Behnke 1992). It is unclear whether historically present Rio Grande cutthroat trout were displaced by introduced *O. c. bouvieri*, or whether *O. c. virginalis* never actually existed at this site.

Results presented in this paper also confirm that the *O. c. virginalis* populations in Cabresto Creek, Cabresto Lake Fork, Cow Creek and Holden Prong all contain substantial amounts of non-native genetic material, and that the population in Middle Ponil is also introgressed. Three of these populations were already known to be hybridized from morphometric or allozyme studies. We suggest that Cow Creek and Holden Prong be re-assigned into the 'Sportfish' category, while Cabresto Lake Fork should be removed from the 'Core' category. It also appears that there is some genetic material derived from *O. c. bouvieri* present in several populations currently classified as 'Core Conservation' populations, in particular Rito del Padre and Rio de las Vacas, and probably also Doctor Creek. Such a result is not surprising: despite extensive stocking of Yellowstone cutthroat trout into New Mexico waters, no study has previously quantified introgression from this taxon into native *O. c. virginalis* and current genetic management categories are assigned on the basis of hybridization with *O. mykiss* only.

Encouragingly, at least six populations whose genetic status was previously unknown or doubtful (DAL, EST, MOR, MTR, OSH, UTE), and six populations currently assigned to the 'Core Conservation' category (BIT, CAN, COL, ELR, PVA, RIC, VDZ, VJO) appear to contain little or no non-native introgression on the basis of our microsatellite data. We also find little evidence for non-native genetic material in the sample from Angostura Creek, previously classified as a 'Sportfish' population, probably because the sample analysed here was taken from a population in the upper stream reaches that was yet to be impacted by the hybrid individuals downstream. It is notable that the genetic integrity of these populations has largely been retained despite records indicating previous stocking of non-natives into many of these streams (Table 1). In many cases this may be because stocking occurred below a migration barrier that protected upstream populations of *O. c. virginalis*. However it may also indicate poor survival or reproduction of introduced hatchery-reared fry. A similar pattern, where the native gene pool has been largely retained despite the intensive stocking of genetically divergent hatchery strains, has been observed in some studies of European brown trout (García-Marín et al. 1998; Heggnes et al. 2002).

In contrast to results presented by Hitt et al. (2003) and Rubidge and Taylor (2005), who noted rapid spread of rainbow trout alleles into previously pure populations of westslope cutthroat trout (*O. c. lewisi*), we found little evidence for recent *O. mykiss* introgression into populations that previously tested free of genetic material from this taxon. Many extant *O. c. virginalis* populations in New Mexico are protected from non-native invasion by the presence of natural or artificial migration barriers, some of which have been constructed specifically to conserve 'Core' populations, and these appear to have limited the further spread of introgression despite ongoing introduction of non-native *Oncorhynchus* into adjacent waters. In contrast, the observation of significant linkage disequilibrium within samples from Cabresto Creek, Cow Creek and Middle Ponil, three streams not currently protected as 'Core Conservation' populations, suggests that non-native introgression into these populations may be recent or ongoing.

Identification and management of introgressed populations within threatened taxa

Management of introgression within taxa of conservation concern is the subject of ongoing political and scientific debate (O'Brien and Mayr 1991; Allendorf et al. 2004, 2005; Campton and Kaeding 2005). There is continuing controversy in many countries regarding the status of hybrid individuals and populations under legislation intended to protect threatened taxa. In Europe, for example, the legal protection of the Scottish wildcat (*Felis silvestris*) is ineffective due to difficulties in discriminating pure individuals from those containing genetic material from the domestic cat (Beaumont et al. 2001), and in the United States similar issues have bedevilled the legal protection of, amongst other taxa, wolves (*Canus* spp., Fredrickson and Hedrick 2006; Kyle et al. 2006) and spotted owls (*Strix occidentalis*, Haig et al. 2004). The situation is exemplified by the contrasting criteria used by the U.S. Fish and Wildlife Service (USFWS) to enumerate populations of three different cutthroat trout subspecies recently rejected for protection under the U.S. Endangered Species Act. When considering the status of the westslope cutthroat trout, *O. c. lewisi*, the USFWS included all populations containing < 20% non-native introgression within the subspecies, on the basis that such populations were phenotypically and behaviourally indistinguishable from pure populations (Department of the Interior 2003). A similar status review for *O. c. bouvieri* included all populations in the 'Conservation' category (generally < 10% introgression), whilst the status review for *O. c. virginalis* included only populations in the 'Core Conservation' category (< 1% introgression, Department of the Interior 2002, 2006). In some cases, the level of introgression which a threatened population is estimated to contain can overwhelmingly influence that population's fate. For example, under the guidance of the Position Paper on cutthroat trout management, populations within either the 'Conservation' or 'Sportfish' category may be eliminated and replaced with wild or broodstock fish originating from populations within the 'Core' category. Similarly, populations of the federally listed Gila trout (*O. gilae*) containing 'high percentages of hybrid individuals' are not managed under the Endangered Species Act and may be destroyed (Porath and Nielsen 2003).

In such a context, it is important that the assessment of introgression levels in a population of conservation concern be as accurate as possible. This can be a difficult task, even in cases where co-dominant, diagnostic markers are available to discriminate the taxa of interest. For example, an admixture estimate can be biased by the sampling scheme: many taxa exhibit intra-population structuring or non-random association of kin, hence a genetic sample taken from a restricted area or a single age-group may be unrepresentative of the population as a whole (Hansen et al. 1997). Furthermore, alleles which appear to be diagnostic in limited reference samples may

appear to become non-diagnostic as number of tested individuals increases, in which case judgement as to whether a shared allele represents recent hybridization or ancestral polymorphism may be largely subjective. As an additional complication, recommendations regarding the management of introgression in threatened taxa may be open to alternative interpretations. In the case of cutthroat trout, it appears that the 'Core Conservation' category, as defined in the Position Paper (Anonymous 2000), was originally intended only to include those populations with no detectable introgression: that is, no non-native alleles are observed in the population sample, and the sample is of sufficient size that the upper confidence limit of the population-wide estimate of introgression is less than 1%. However, in some cases it appears that management agencies include populations in this category when the introgression level observed in the sample is less than 1%, irrespective of the confidence limits of the population estimate. As there is no theoretical reason to suppose that a population containing 0.9% genetic material from non-natives is at any less risk from inbreeding depression than one containing 1.5% genetic material, this approach renders the distinction between 'Core Conservation and 'Conservation' populations essentially arbitrary. Finally we note that the relationship between admixture levels as estimated from neutral genetic markers, and the proportion of coding non-native genome within the native form, is poorly understood.

In this paper, we have demonstrated that, within a taxon of conservation concern, the estimated level of non-native admixture, based on data from microsatellite loci with overlapping allele size distributions between the putative parental forms, can vary both with the statistical approach used and the reference samples provided. Although we have not explicitly addressed the following issues, we would also expect a population-level admixture estimate to vary between different samples collected from the same stream, and with factors such as the sampling scheme implemented, the size of the sample collected and the number of loci used. In some cases, decisions regarding the binning of microsatellite alleles or the pooling of reference samples may also affect results. Clearly, when using such non-diagnostic and highly variable genetic markers to estimate admixture, it is vitally important to compare results from different analytical approaches and using different parental references before coming to any conclusion about the hybrid condition of a population. In cases where management agencies wish to unambiguously assign populations to management categories based on threshold levels of introgression, continuing efforts to develop diagnostic markers would be warranted. Irrespective of whether or not available markers are diagnostic, accurate assessment of the level of introgression in a population also requires a carefully designed sampling scheme. We urge further debate on the implications of assigning populations to such rigidly defined management categories. In particular, we note that populations of threatened taxa containing some non-native genetic material may also contain native genetic diversity not present in other populations. Hence the rationale for eliminating these populations and replacing them with 'pure' stock should be carefully considered, especially in cases where assessment of introgression levels is difficult and the definition of stocks considered 'pure' is open to alternative interpretations.

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