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Original Article

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Limited, asymmetric hybridization between coastal cutthroat trout and steelhead in a Northern California river

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Abstract

Hybridization between coastal cutthroat trout (*Oncorhynchus clarkii clarkii*) and steelhead (*O. mykiss*) was assessed in the Smith River, California. Individuals were categorized as pure or as 1 of 10 hybrid classes using 30 "diagnostic" single-nucleotide polymorphisms positioned on 26 separate chromosomes. Most of the individuals examined ($n = 876$), were pure coastal cutthroat trout ($n = 634$) or pure steelhead ($n = 213$), and 29 individuals were identified as having hybrid ancestry. Among hybrids, first generation hybrids ($n = 15$) and coastal cutthroat trout backcrosses ($n = 12$) were the most common. No individuals were identified as backcrosses to SH, suggesting the presence of genetic or behavioral mechanisms constraining such backcrosses, or the growth and survival of their progeny. Mitochondrial DNA of 14 of 15 F1 hybrids was of steelhead origin, suggesting that hybridization was driven primarily by sneak-mating of male coastal cutthroat trout with female steelhead. Evaluation of classical phenotypic characters for coastal cutthroat trout and steelhead (i.e. jaw slash, maxillary length, and hyoid teeth) were not reliable by themselves for identification of either pure parental fish or hybrids. In contrast, analysis with geometric morphometrics revealed distinctive body shapes for pure coastal cutthroat trout and steelhead, and the combination of classical traits and geometric morphology was mostly accurate in distinguishing them. However, first generation hybrids and backcrosses overlapped completely with parental types, highlighting challenges in hybrid identification using phenotypic traits.

Key words: asymmetric hybridization, coastal cutthroat trout, geometric morphometrics, steelhead

Introduction

Closely related species living in sympatry usually fill distinct niches related to environment, morphology, and behavior (Kozfkay et al. 2007; Hasselman et al. 2014; Pacheco-Sierra et al. 2016). Interactions among related sympatric species are tempered by divergent traits that act as isolating mechanisms, limiting gene flow (Abbott et al. 2013; Harrison and Larson 2014). However, if isolation is incomplete, occasional hybridization and gene exchange may occur, creating a dynamic balance between the production of hybrids and their removal by natural selection (Taylor et al. 2006). Understanding physical, ecological, and genetic factors that mediate the balance between hybridization and continued divergence of sympatric species is integral to informed species management.

Hybridization between distinct species is rare, due to the presence of pre- and postzygotic isolating mechanisms, such as spatial/temporal isolation or reduced hybrid fitness (Mallet 2005). Nonetheless, hybridization between diverging species, when it occurs, is a necessary first step for interspecific gene exchange. The next step in interspecific gene exchange is backcrossing—mating between a mixed-ancestry hybrid and a pure member of 1 parental species. Backcrossing can become more common than hybridization between the parental species, over time, since it produces offspring each generation that are more genetically similar to 1 parental species, which may lessen the effect of isolating mechanisms (Goodman et al. 1999).

Hybridization presents a window into the process of incipient speciation (Barton and Hewitt 1985). Thousands of generations of environmental variation and natural selection in natural hybrid zones create a complexity of hybrid genotypes unmatched in laboratory settings (Rieseberg et al. 1999). Natural hybridization and backcrossing introduce novel gene combinations, but whether they result in outbreeding depression, adaptive advantage, or are simply byproducts of the process of speciation varies widely with the details of the hybridizing taxa (Barton and Hewitt 1985; Rieseberg et al. 1999; Abbott et al. 2013). Recent advances in genetic techniques have opened the door to detailed observation of hybrid genomes, and enabled research on natural hybridization to explore stability of hybrid zones on a case-by-case basis and better understand the process of speciation.

Coastal cutthroat trout (*Oncorhynchus clarkii clarkii*, CCT) and coastal steelhead (*O. mykiss irideus*, SH) are

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presumed to have shared a long evolutionary history of sympatry (Campton and Utter 1985). Molecular phylogenetic analysis and time-dated trees suggest that the 2 sister species (*O. mykiss* ssp. and *O. clarkii* ssp.) diverged from a common ancestor about 3 to 10 million years ago (Wilson and Turner 2009; Crête-Lafrenière et al. 2012). The native range of CCT, which extends from Northern California, United States to Southern Alaska, United States (Trotter 1989), is almost completely overlapped by that of SH, but hybridization between the 2 species occurs predominantly where spawning habitat overlaps (Campton and Utter 1985; Buehrens et al. 2013). Relatively recent divergence on an evolutionary time scale (Coyne and Orr 1997; Mallet 2005) and reproduction by external fertilization (Scribner et al. 2000) are believed to have brought about favorable conditions for natural hybridization between CCT and SH.

The areas where CCT and SH hybridize encompass a vast geographical area featuring regional environmental variation and chromosomal divergence between CCT and SH populations (Gold et al. 1977; Thorgaard 1983). Morphologic divergence between CCT and SH has been strongly linked to their distinctive life histories. CCT have elongate bodies, large mouths, and piscivorous feeding habits as adults. They spawn in headwater tributaries at a fork length (FL) of 300 to 500 mm and, when anadromous, undertake short ocean or estuary migrations (Trotter 1989). SH, in contrast, are larger and deeper bodied and feed primarily on macroinvertebrate drift in fresh water. They typically spawn lower in the watershed than CCT at a FL of 600 to 800 mm, and are known for their long ocean migrations of hundreds to thousands of kilometers over 1 to 3 yr (Withler 1966). Population-level hybridization in different locales throughout the CCT and SH sympatric ranges has produced highly variable outcomes, ranging from hybrid swarms with extensive backcrossing (Bettles et al. 2005), to asymmetric hybridization (Baumsteiger et al. 2005), to near-complete reproductive isolation (Young et al. 2001).

In this study, we applied 30 "diagnostic," single-nucleotide polymorphisms (SNPs) on separate chromosomes or separate arms of the same chromosome to characterize the extent and geographic distribution of CCT/SH hybridization in the Smith River Basin of California, United States, where they are in secondary contact. Additionally, geometric morphology data were paired with the genetic data to assess the potential for identification of individuals from hybrid classes with morphometrics. The use of a large panel of diagnostic markers located across the genome, coupled with extensive collections from both the main tributaries and the estuary, allowed for detailed characterization of hybridization at the population and individual level, as well as across size categories of fish. We examine the extent to which these 2 sympatric species are likely to form a hybrid swarm, compromising the integrity of the 2 parental species, or whether hybridization in this near-pristine river is limited and commensurate with predictions of speciation theory.

Methods

Study site

The Smith River drains a 1,950 km^2 basin of steep forested terrain in the western Klamath and Siskiyou Mountains in Northern California and Southern Oregon, United States. Designated as a National Wild and Scenic River, it is the largest free-flowing river in the region and is renowned for its water clarity and rugged nature, and provides 395 km of habitat for CCT. In addition to CCT and SH, the Smith River also supports Chinook Salmon (*O. tshawytscha*), and Coho Salmon (*O. kisutch*) populations. To spatially distribute collections, we sampled fish in 7 river sections: 1) the estuary (the mouth of the Smith River to Morrison Slough), 2) Rowdy Creek, 3) the mainstem Smith River (Morrison Slough to the confluence of the South and Middle Forks of the Smith River), 4) Mill Creek, 5) the South Fork Smith River, 6) the North Fork Smith River, and 7) the Middle Fork Smith River (Fig. 1). One of the sampled tributaries of the Middle Fork Smith River, Little Jones Creek, is above a barrier to anadromy.

Field collections

Fish were captured using night netting, hook and line, electrofishing, and weirs from mid-May through August, 2013. Night netting provided an effective capture technique, in which divers used underwater lights to temporarily disorient fish while a hand net was raised slowly underneath them. Within each of the 7 subbasins, we sought sample sizes of 130 trout (CCT, $n = 100$; SH, $n = 30$) with FL > 100mm. Unequal sampling of CCT compared with SH was used to assess fine-scale population structure within CCT, an ancillary research goal. We targeted fish expected to be at least yearlings (FL > 100mm), as species identification between CCT and SH is more challenging for juvenile trout in their first year of development, and also to decrease the probability of capturing closely related individuals by allowing sufficient time for siblings to disperse from natal areas (Buehrens et al. 2013). A tissue sample, photograph, and FL measurement were collected from 884 fish. Phenotypic characteristics of jaw slash color intensity, maxillary length compared with posterior margin of eye, and presence of hyoid teeth were also recorded for each fish in the field (Kennedy et al. 2009). Jaw slash color intensity was scored as not present—0, faint—1, or bright—2. Maxillary lengths were ranked as shorter than posterior margin of eye—0, extends to posterior margin of the eye—1, or beyond the posterior margin of the eye—2. The absence of hyoid teeth was scored as—0, and the presence of hyoid teeth as—1. Fish with mouths too small to check for hyoid teeth were scored as—NA.

Molecular methods

DNA was extracted from tissue samples using DNeasy 96 Blood and Tissue Kits, with a protocol modified for use on a BioRobot 3000 workstation (Qiagen Inc.). We generated genotypes for 96 SNPs using TaqMan 5′ nuclease assays (Applied Biosystems Inc.) and 96.96 Dynamic Genotyping Arrays (Fluidigm Corporation), with imaging on an EP1 instrument l (Fluidigm). Genotypes were manually called using the SNP Genotyping Analysis Software (3.0.2, Fluidigm).

We first surveyed variation at 96 SNP loci, including 65 loci that were previously identified as fixed or nearly fixed for alternate alleles in CCT and SH (64 loci from Pritchard et al. 2012; 1 from Campbell et al. 2012) and 31 loci that were polymorphic in CCT (Pritchard and Garza 2013). A frequency threshold of >0.98 for one of the alleles in both the CCT and northern SH populations surveyed by Pritchard et

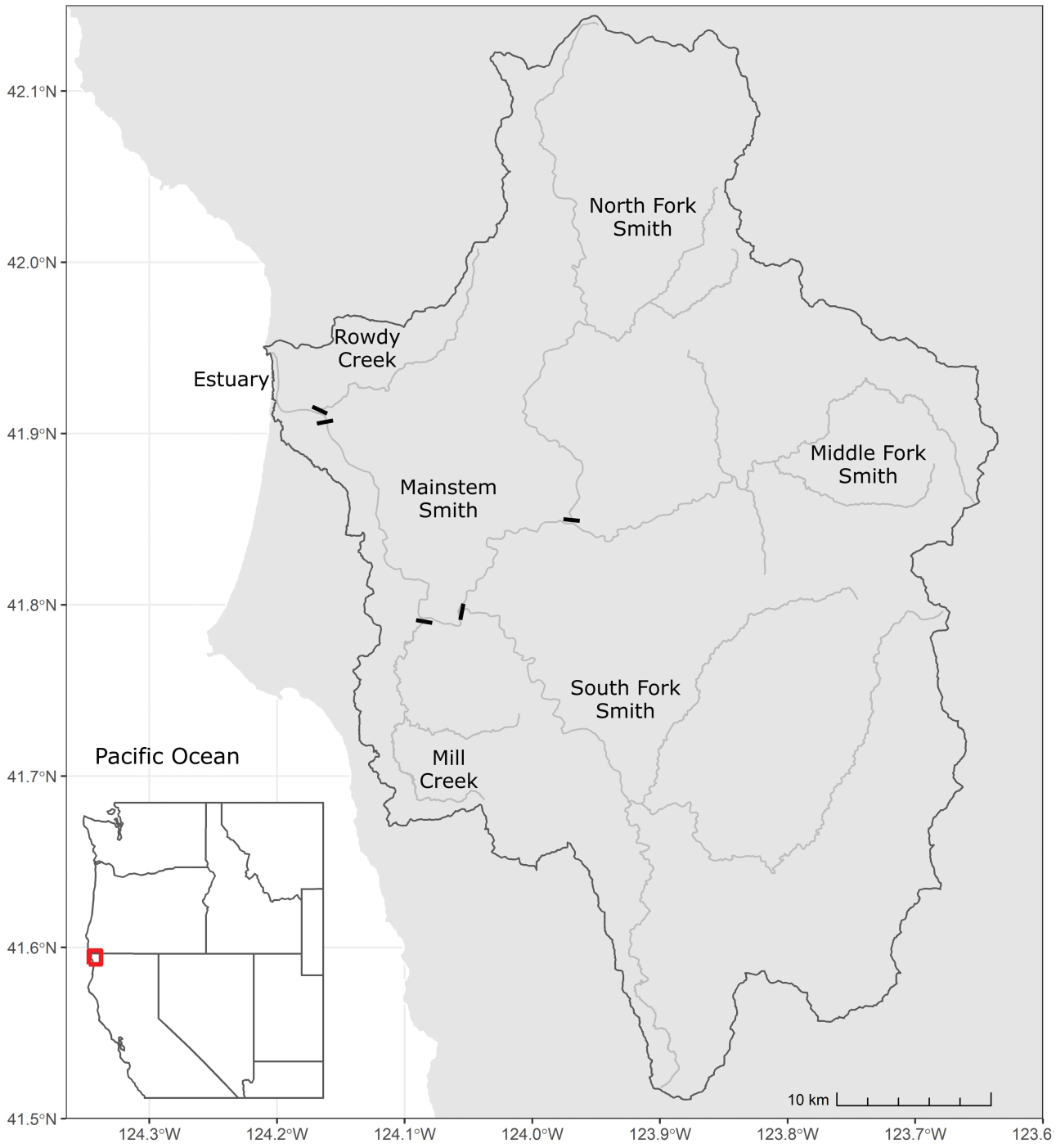

Fig. 1. Subbasins of the Smith River, California, sampled in 2013 for coastal cutthroat trout, steelhead, and their hybrids. Subbasins are labeled, and boundaries are marked with a black bar, perpendicular to flow. Inset shows location of study site in United States.

al. (2012) was used to identify potentially diagnostic loci in the Smith River. Loci that were polymorphic in the southern SH populations from Pritchard et al. (2012), outside the range of CCT, were not considered diagnostic due to the inability to disentangle gene flow from ancestral polymorphism. We mapped the positions of the 65 potentially diagnostic loci using the *O. mykiss* genome, Omyk_1.0 (Gao et al. 2018), using Bowtie2 (Langmead and Salzberg 2012) (Fig. 2). Of these 65 loci, 30 were located on distinct chromosomes or separate arms along the same chromosome, with markers on 26 *O. mykiss* chromosomes, so they could be treated as independently segregating markers. These 30 loci were used to determine an individual's hybrid class using NewHybrids (see below). Individuals were removed from analyses if genotypes were missing at >20% of the loci, leaving genotypes from 876 fish available for analysis.

The mitochondrial gene, NADH dehydrogenase-1 (ND-1), was used to identify the maternal lineage of first generation hybrids (F1). Mitochondrial haplotypes indicative of either SH or CCT were identified following procedures described in Baumsteiger et al. (2005) and Baker et al. (2002).

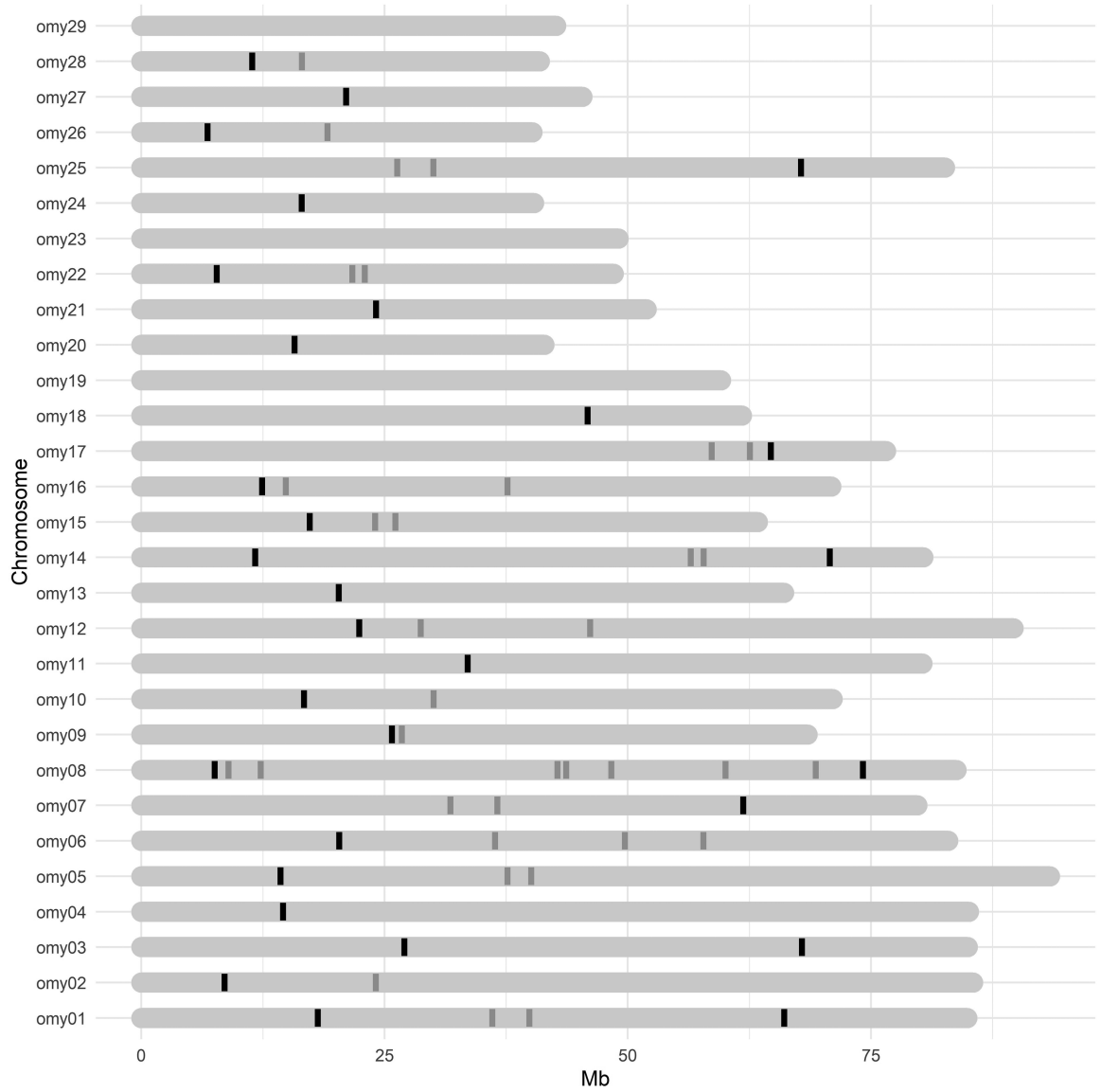

Fig. 2. Locations of 65 loci on the 29 chromosomes of the steelhead genome. Vertical lines represent the location of loci on the chromosome (omy01, omy02, etc.). The black vertical lines represent the 30 loci used in the NewHybrids analysis to determine hybrid class.

Hybrid analysis

Hybrid class was determined using the 30 independently segregating "diagnostic" loci with the software NewHybrids (Anderson and Thompson 2002; https://github.com/eriqande/newhybrids/tree/6fc8fd9c). NewHybrids was configured to assign fish to 10 hybrid classes characterized by the expected frequencies of loci having 0, 1, or 2 alleles originating from each parental type (Supplementary Table S1). Five independent Markov chain Monte Carlo simulations of 50,000 iterations, with a burn-in of 10,000 iterations, were run and the results compared with confirm convergence. Individuals were assigned to a hybrid class if their posterior probability for that class was >0.85.

Morphological analysis

Body morphologies of CCT, SH, and their hybrids were compared using landmark-based geometric morphometrics. Unlike traditional morphometrics, which uses angles and distances between morphological attributes, geometric morphometrics uses the relationship between all the landmarks to represent the shape of an individual (Zelditch et al. 2012), providing more power in the discrimination of species (Maderbacher et al. 2008). Landmarks, which are discrete anatomical locations found in all individuals, were identified and recorded on the digital photographs taken of each fish. Fourteen landmarks were chosen, based on previous geometric morphometric applications on salmonids and known morphometric differences between CCT and SH (Kennedy et al. 2009; Varian and Nichols 2010; Stelkens et al. 2012). Landmark locations were as follows: 1) tip of snout; 2) anterior margin of eye; 3) posterior margin of eye; 4) posterior end of operculum; 5) posterior end of maxillary; 6) origin of pectoral fin; 7) origin of pelvic fin; 8) origin of anal fin; 9) anterior attachment of ventral membrane of caudal fin; 10) base of middle caudal rays; 11) anterior attachment of dorsal membrane of caudal fin; 12) origin of adipose fin; 13) origin of dorsal fin; and 14) posterior end of the neurocranium (Supplementary Fig. S1). To ensure landmark placement did

not migrate, and to adjust for imperfect digitizing, each fish was landmarked twice and results of the digitizing events were averaged (Klingenberg 2011).

Individuals were not included in the geometric morphometric analysis if the associated image was not taken from directly above the fish, the mouth of the fish was open, the caudal peduncle was not in line with the rest of the body, or image quality was poor. Landmarks were identified and their locations digitized on the images using tpsDIG2 (Rohlf 2010). A scale factor was calculated for each image to account for differences in focal length among photographs. A known-length scale bar was included in each photograph and then converted into pixels to calculate the scale factor.

As morphology varies with ontogeny (Klingenberg 1998), small fish (FL ≤ 200 mm, $n = 290$) and large fish (FL > 200 mm, $n = 275$) were separately analyzed. A break at 200 mm FL was chosen to account for smoltification, a process with the potential to influence body morphology, primarily occurring in CCT and SH between 100 and 200 mm FL (Trotter 1989; Ward et al. 1989). A Procrustes superimposition was applied to minimize the sum of squared distances between landmarks. Procrustes superimposition removes non-shape variation from the data on landmark coordinates using scaling, rotation, and translation to compare individuals to a mean consensus shape. The Procrustes coordinates were plotted against the log of the centroid size for each individual, with the residuals used in all further analyses to account for the effects of allometry. The morphometric residuals and phenotype scores from slash intensity and presence/absence of hyoid teeth were used first in a factor analysis of mixed data, then these scores were applied to a linear discriminant analysis (LDA) to identify the major axes of shape and phenotype variation (Venables et al. 2002; Le et al. 2008; Kuhn 2021). Scores from the first 2 LDAs were plotted to visually inspect shape and phenotype differences among the genetically determined hybrid classes for both the small and large size classes separately. All morphometric analyses were performed in Morpho J (Klingenberg 2011). All other analyses were performed using R Statistical Software (4.1.2; R Core Team 2021), unless otherwise stated.

Results

Hybridization

Most individuals were identified as pure CCT ($n = 634$) or pure SH ($n = 213$). Of 29 individuals identified as having hybrid ancestry, most were F1 hybrids ($n = 15$). The remaining individuals included 1 second generation hybrid (F2), 12 first (BxC) or second generation CCT backcrosses (BxC2), and 1 individual likely of a more complex hybrid class (Table 1). Not every hybrid individual had genotypes conforming exactly to expectations, because the majority of our markers were not completely fixed for alternative alleles in the parental species, presumably as a consequence of historic hybridization and genotyping error. All but 1 individual was assigned to a hybrid class with a posterior probability >0.85. The 1 uncertain sample was equally likely to have been either an F2 or the result of a BxC and BxC2 mating. Our data were not sufficient to resolve those cases, and ultimately this individual was classified as a FNxBxC. Hybrid analysis with all 65 of the potentially diagnostic markers produced identical results, but we report the 30-marker results here because of the strong linkage between many of these markers.

The absence of SH backcrosses and the occurrence of individuals from multiple CCT backcross generations showed that backcrossing rates to the 2 parentals were not equal ($P = 0.0002$, Binomial test), indicating asymmetric hybridization (Fig. 3). To confirm that this result was not due to our sampling design that intentionally yielded more field-identified CCT than field-identified SH, we also performed a Fisher's exact test comparing the number of backcrosses as a fraction of the number of field-identified SH and CCT, using

Table 1. Number of individuals in each hybrid class, by size class (≤200 and >200 mm) and river section.

Hybrid class	Size class	Estuary	Rowdy Creek	Mainstem	Mill Creek	SF Smith	NF Smith	MF Smith
SH	>200mm	2	8	5	1	14	7	20
F1		2	3	1	0	1	0	0
F2		0	0	0	0	0	0	0
FNxBxC		0	0	1	0	0	0	0
BxC		0	0	0	0	0	0	0
BxC2		0	0	0	0	0	0	0
CCT		62	22	56	17	59	65	62
Total		66	33	63	18	74	72	82
SH	≤200mm	28	14	24	31	18	23	18
F1		5	0	0	0	2	0	1
F2		1	0	0	0	0	0	0
FNxBxC		0	0	0	0	0	0	0
BxC		2	0	0	3	0	1	0
BxC2		0	0	1	2	0	0	3
CCT		23	33	16	97	39	15	68
Total		59	47	41	133	59	39	90

MF, Middle Fork; NF, North Fork; SF, South Fork. Hybrid classes are defined as follows: CCT: pure coastal cutthroat trout; SH: pure steelhead; F1: first generation hybrid; F2: second generation hybrid (F1xF1); FNxBxC: either an F2 or cross between BxC and BxC2; BxC: cross between F1 and CCT; BxC2: cross between BxC and CCT.

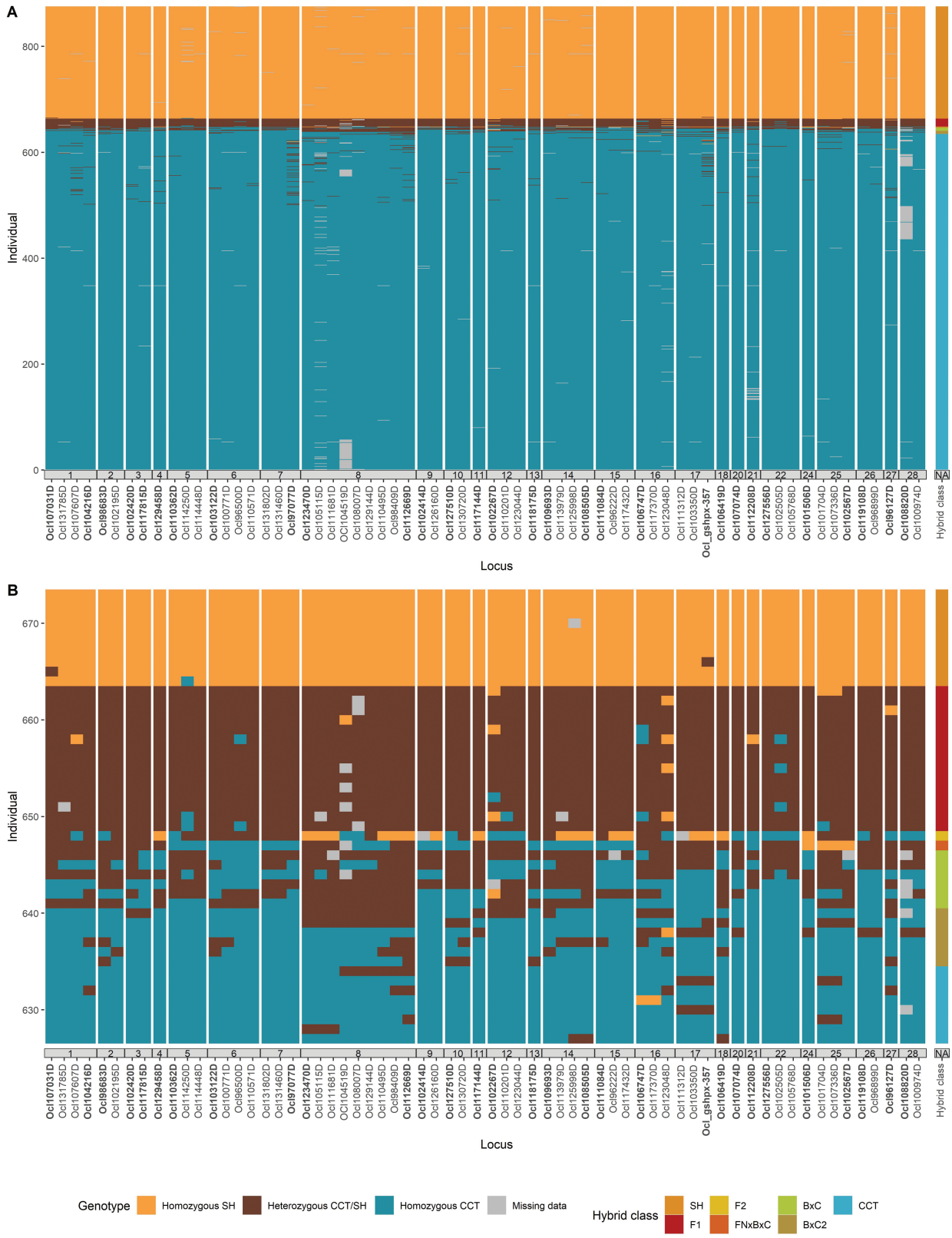

Fig. 3. Genotypes for 65 loci in the 876 individuals examined. Each individual occupies a row, each column a locus (listed according to their mapped order in the genome) and the individual cells are colored according to genotype. Vertical white lines separate chromosomes, whose numbers appear in the boxes at the bottom of the figure. The column at the far right gives the inferred hybrid class of each individual. Locus labels were made bold if they were used in the 30-marker hybrid analysis. A) All individuals sorted from pure steelhead (top) to pure coastal cutthroat (bottom). B) Detailed view of 48 individuals, which includes those identified as hybrids. Hybrid classes are defined as follows: CCT: pure coastal cutthroat trout; SH: pure steelhead; F1: first generation hybrid; F2: second generation hybrid (F1xF1); FNxBxC: either an F2 or cross between BxC and BxC2; BxC: cross between F1 and CCT; BxC2: cross between BxC and CCT.

the observed counts: 13 of 646 field-identified CCT were genetic backcrosses, versus 0 of 214 field-identified SH. This demonstrated that the occurrence of hybrids in our study is not independent of field-identified species, but a consequence of asymmetric hybridization ($P = 0.0461$).

Individuals of hybrid ancestry (F1, F2, FNxBxC, BxC, or BxC2) were found within all 7 river sections (Table 1). Small fish (<200 mm FL) of hybrid ancestry were also identified in all river sections, except Rowdy Creek, indicating the production of hybrid individuals likely occurs in most subbasins. All individuals examined from Little Jones Creek, which is isolated above a natural barrier to anadromy, were identified as pure CCT.

All first and second generation CCT backcrosses were small at capture (Table 1; Supplementary Fig. S2). The BxC ($n = 6$) and BxC2 ($n = 6$) individuals ranged from 114 to 173 mm FL (mean 142 mm). In contrast, the 15 F1 individuals ranged from 153 to 401 mm FL (mean 242 mm) and pure CCT ranged from 102 to 438 mm FL (mean 227 mm) and pure SH ranged from 112 to 535 mm FL (mean 190 mm).

Analysis of mitochondrial DNA in 15 individuals identified as F1 hybrids revealed that 14 had mtDNA from SH, indicating initial matings between the parental species were almost exclusively between SH females and CCT males.

Morphology

Although there were some differences between species in the frequencies of the 3 morphological trait values, both when considered separately (Table 2) and in combination (Supplementary Tables S2 and S3), the absence of diagnostic patterns typically associated with CCT and SH identification (Kennedy et al. 2009), did not allow hybrids to be readily distinguished in the field. While the combination of all 3 traits was nearly diagnostic for parentals of the 2 species (Supplementary Table S3), it was largely because so many fish were eliminated due to the difficulty of non-lethally evaluating the hyoid teeth trait in many fish. The geometric morphometric analysis using data from the 565 individuals with a genotype and appropriate photograph, revealed that CCT and SH differed primarily with respect to body depth, snout length, and maxillary length (Supplementary Fig. S3). CCT generally had shallower body depth, longer snout, and longer maxillary compared with SH.

After combining body morphology, slash, and hyoid teeth presence/absence in the LDA analysis, both small and large CCT and SH had nearly nonoverlapping distributions, but individuals of hybrid ancestry were not distinguishable from the parental species (Fig. 4). Notably, F1 and F2 hybrids were largely intermediate between CCT and SH, and backcrosses were indistinguishable from CCT (Supplementary Figs. S4 and S5).

Discussion

Genetic evaluation of populations of CCT and SH in the Smith River Basin of California, United States allowed a detailed view of reproductive interactions between these sister species and found strong evidence for limited hybridization in all the river sections of the study area. Moreover, this hybridization was highly asymmetrical; F1 hybrids were almost exclusively the product of a male CCT mating with a female SH. Furthermore, SH backcrosses were absent, but CCT backcrosses were one of the most common types of hybrids identified. The natural, sympatric occurrence of CCT and SH contrasts with that of the inland cutthroat trout subspecies, where sympatry is almost always the result of *O. mykiss* introduction (Allendorf et al. 2001). Whereas inland trout in sympatry frequently result in hybrid swarms and loss of pure parental fish, the limited number of hybrids observed in the Smith River, despite their ability to produce viable and fertile

Table 2. Number of individuals in each hybrid class by size class (≤200 and >200 mm), slash intensity, maxillary length, and presence/absence of hyoid teeth.

		Slash			Maxillary			Hyoid		
Hybrid class	**Size class**	**0**	**1**	**2**	**0**	**1**	**2**	**0**	**1**	**NA**
SH	>200 mm	56	1	0	13	23	21	55	1	1
F1		4	3	0	0	0	7	5	2	0
F2		0	0	0	0	0	0	0	0	0
FNxBxC		1	0	0	0	0	1	0	1	0
BxC		0	0	0	0	0	0	0	0	0
BxC2		0	0	0	0	0	0	0	0	0
CCT		65	188	92	0	2	341	13	316	14
SH	≤200 mm	156	0	0	133	22	1	6	0	150
F1		7	1	0	1	1	6	3	3	2
F2		1	0	0	0	0	1	1	0	0
FNxBxC		0	0	0	0	0	0	0	0	0
BxC		1	5	0	0	3	3	0	1	5
BxC2		1	2	3	0	2	4	0	3	3
CCT		8	137	146	0	25	266	13	153	125

See Methods for definitions of trait categories. Hybrid classes are defined as follows: CCT: pure coastal cutthroat trout; SH: pure steelhead; F1: first generation hybrid; F2: second generation hybrid (F1xF1); FNxBxC: either an F2 or cross between BxC and BxC2; BxC: cross between F1 and CCT; BxC2: cross between BxC and CCT.

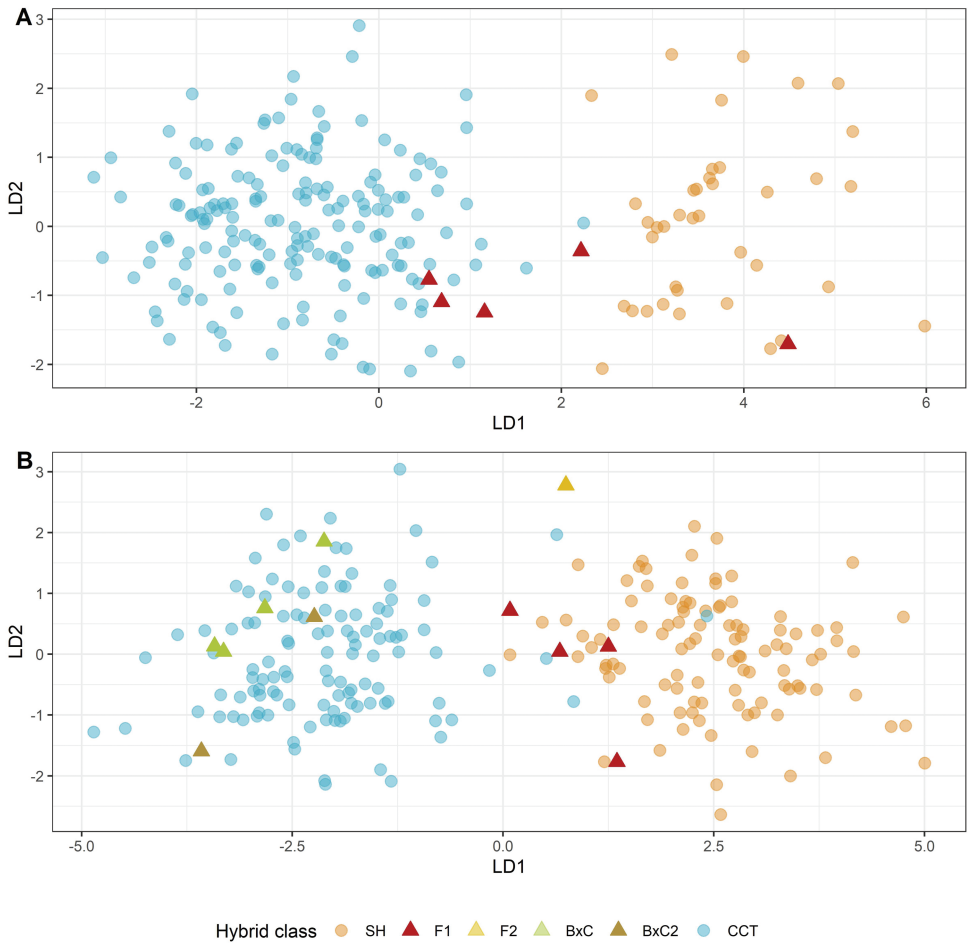

Fig. 4. A) LDA scores of the first 2 axes, combining geometric morphology and phenotype for large trout (FL > 200 mm; $n = 275$) and B) small trout (FL ≤ 200 mm; $n = 290$). Hybrid class, as defined by the NewHybrids analysis, is indicated by the circles for the parental types and triangles for the hybrid individuals. The single CCT individual in the SH point cloud did not have hyoid teeth. Hybrid classes are defined as follows: CCT: pure coastal cutthroat trout; SH: pure steelhead; F1: first generation hybrid; F2: second generation hybrid (F1xF1); FNxBxC: either an F2 or cross between BxC and BxC2; BxC: cross between F1 and CCT; BxC2: cross between BxC and CCT.

hybrid offspring, suggest substantial barriers to hybridization. This is presumably due to a long evolutionary history of interactions and points to intrinsic and extrinsic ecological and behavioral mechanisms that maintain partial reproductive isolation (Campton and Utter 1985; Ostberg et al. 2004; Buehrens et al. 2013).

Asymmetric hybridization

While the direct causes of asymmetric hybridization are uncertain, factors including mating behavior, fitness differences, and variation in population density can produce a directional effect (Barton and Hewitt 1985; Ostberg et al. 2004). The observation of F1 hybrids from both size classes, and with FLs ranging from 150 to 401 mm, strongly suggests multiple year classes are present, indicating ongoing mating between SH and CCT. Nearly all the F1 hybrids carried mtDNA from SH, indicating that hybridization between the parental species primarily occurred as a result of matings between SH females and CCT males, consistent with previous studies (Ostberg et al. 2004; Baumsteiger et al. 2005). Ostberg et al. (2004) proposed 2 explanations for asymmetric hybridization between CCT and SH: 1) a male CCT sneak-mating strategy with female SH and 2) weakened assortative mating, whereby female SH "settle" for a male CCT, due to the absence or rarity of male SH (Wirtz 1999). Sneak-mating strategies result from size-based spawning hierarchies, in which smaller males attract less attention from larger aggressive males while fertilizing eggs (Fleming 1998).

Hawkins and Foote (1998) found that egg size of CCT and SH offspring, spawned in a laboratory setting, depended on parental type of the dam. Hybrid offspring with SH dams emerged earlier and had a larger yolk sac than did hybrids with CCT dams. Although survival did not differ between hybrid offspring types in the laboratory setting, the head start experienced by hybrid offspring of SH dams may produce an immediate growth advantage in the wild, providing a possible explanation for asymmetry in F1 hybridization.

Morphological differences between SH (Withler 1966) and CCT (Trotter 1989) may coincide with life history (ocean versus estuary migration) and habitat preferences (fast versus slow water). Hawkins and Quinn (1996) found juvenile hybrid individuals held a competitive advantage over CCT, a possible fitness-related explanation for asymmetric introgression of SH alleles into CCT. Furthermore, decreased fitness of F1 hybrids and backcrossed SH attempting taxing ocean migrations may increase mortality resulting in a reduced presence of these hybrid types (Ostberg et al. 2004). Moore et

al. (2010) reported that acoustic tagged juvenile CCT, SH, and their hybrids displayed distinct migration patterns within Puget Sound, with hybrids traveling an intermediate distance, farther than CCT but truncated in comparison to SH. However, variation in ocean migration patterns among CCT, SH, and their hybrids is not well studied.

Fitness differences between 2 species can also contribute to differences in the frequencies of the observed backcrosses. Backcrossing results from reproduction of F1 or other hybrid classes with parental individuals. In the absence of selection or assortative mating, a hybrid population would exhibit first generation backcrosses at twice the proportion of F1s (Goodman et al. 1999). The absence of SH backcrosses suggests either strong assortative mating or intense selection at an early life stage on these backcrosses. Likewise, the absence of BxC and BxC2 that were larger in size suggests the presence of genetic or behavioral mechanisms limiting the growth or survival of CCT backcrosses.

Differential introgression

In the face of hybridization, differential introgression of genomic regions may be as important to maintaining species boundaries as other elements of reproductive isolation, such as prezygotic and exogenous mechanisms. The findings of this study do not allow a definitive characterization of the genomic architecture of hybridization, due to the relatively small number of later-generation hybrids encountered and the limited genomic coverage of the markers employed. Nevertheless, our findings suggest rates of introgression may vary considerably across the genome, as has been observed in many other systems (Gompert and Buerkle 2011; Parchman et al. 2013). In particular, on chromosome 8, the vast majority of backcrosses to CCT carry a SH allele, whereas on chromosome 6, backcrosses vary in the parental source of the alleles (Fig. 3B). Differences in chromosome number between CCT and SH, as well as variation in chromosome number in SH, may result in structural variation that suppresses recombination, and leads to conservation of large blocks of species-specific DNA (Ostberg et al. 2013). Therefore, differences in chromosome number between CCT and SH may facilitate intrinsic genetic isolation through the suppression of recombination. Identification of later-generation hybrids and characterization of variation in those individuals at a much greater density of genomic regions are necessary to elucidate the genomic architecture of hybridization in these species.

Sampling considerations

Differences in the age at capture among previous studies confound the comparison of the incidence of sampled hybrids. If, in fact, hybrids have reduced fitness, their contributions to the spawning stock may be overestimated by primarily sampling the juvenile life stage (Baumsteiger et al. 2005; Kennedy et al. 2009). Our collection of post age 0 and 1+ life stages allowed a more complete life history to play out. Additionally, clustering of juveniles can lead to collections of siblings, which can also affect the estimation of hybrid occurrence (Hansen et al. 1997). Future studies should use a rigorous sampling design that provides a representative sample of both juvenile and adult life stages and avoids localized collections, to more accurately estimate the incidence of hybridization.

Studies may unknowingly mischaracterize the frequency of introgressed individuals, when a small number of markers are analyzed or particular genomic regions are unequally represented in the data (Payseur 2010). Because of these constraints, we were unable to adequately characterize differences in patterns of introgression of specific loci or chromosomal regions. However, our approach of using only genotyped loci from different chromosomes or chromosome arms in the hybrid analysis allowed high confidence in determining an individual's hybrid class, as almost all of the chromosomes were represented. Similarly, individuals of the 10 hybrid classes were separated by no more than 3 generations from a pure CCT ancestor and a pure SH ancestor because, with only 30 independently segregating markers, it is difficult to distinguish between hybrids that are 4 or more generations removed from a pure ancestor and pure individuals carrying rare alleles at loci that may segregate variation from both species. After 3 generations, the expected number of gene copies from the ancestor is less than 2 out of 30, which can result from genotyping errors or previously unidentified shared polymorphism.

The use of genetic markers fixed, or nearly so, for alternative alleles in parental populations without known hybridization, provides benefits but also limitations. Exclusive use of such markers can underestimate the amount of introgression, as loci that have experienced significant previous introgression are excluded. In contrast, including markers that are variable in both species in the hybrid analysis, may overestimate introgression, as such loci may confound ancestral polymorphisms with introgression of CCT alleles into the SH genome (Supplementary Table S4 and Supplementary Fig. S6). Regardless, the choice of which markers to include would not have changed our frequency estimates, or the overall asymmetric pattern, of hybridization.

Morphological analyses

Concordant with previous work (Baumsteiger et al. 2005), juvenile CCT and SH can largely be distinguished by the combination of maxillary length and slash intensity (Supplementary Table S2). However, the relatively small gape of SH makes the identification of hyoid teeth presence/absence problematic for juveniles. Alternatively, the combination of all 3 phenotypic traits can distinguish large (>200 mm) CCT and SH (Supplementary Table S3). Hybrid individuals were indistinguishable from parental types using combinations of phenotypic characteristics, substantiating the difficulty of identifying hybrids in the field (Baumsteiger et al. 2005; Kennedy et al. 2009).

Separation between CCT and SH morphologies, with hybrids exhibiting morphologies overlapping both parental types, is consistent with previous findings (Kennedy et al. 2009). Even post hoc morphometric analysis and a combined analysis of phenotypic traits and body shape could not readily identify F1 hybrids, although the combined analysis showed they were largely intermediate. Similarly, CCT backcrosses occupy a morphospace completely overlapping CCT, suggesting that morphologies of hybrids quickly revert to the dominant parental type with backcrossing. While advances in genetic techniques have led to increasing ability to analyze hybridization and resolve fine-scale introgression (Nolte et al. 2009; Parchman et al. 2013), the associated genomic changes may not translate into easily distinguishable phenotypic expression (Allendorf and Leary 1988).

Understanding mechanisms of hybridization and resulting introgression requires a detailed characterization of the

occurrence of hybrids of different classes and their associated phenotypic characteristics. Here, we provide such an evaluation for SH and CCT populations in the Smith River Basin of California, United States, near the southern end of the sympatric range of the 2 species.

In the face of ongoing hybridization and introgression, genomic regions of reproductive isolation may be as important to maintaining species boundaries as prezygotic and exogenous isolation mechanisms. Continued divergence despite regions of introgression is in accordance with current speciation concepts (Wu 2001).

Conclusion

The implementation of a set of SNP markers developed to identify the 2 parental species, due to fixed or nearly fixed alternative alleles, is key to the accurate assessment of hybridization at both the individual and population levels. Further, a set of markers that provides coverage across the majority of chromosomes allows for accurate determination of hybrid classes. Use of such a set of markers, here, allowed us to accurately identify hybrids and determine that hybridization is highly asymmetric in this river basin. Given that morphometric and phenotypic data can not accurately identify hybrids in this geographic region, the importance of genetic methods for tracking hybridization as well as trends in its distribution and frequency is further highlighted.

Future research into the genomic architecture of CCT and SH hybrids will be aided by a chromosome-level cutthroat trout genome assembly, allowing a more precise analysis of hybridization at the genomic level, as well as the evaluation of hybrids with a much larger and representative set of genetic markers. Such characterization will then facilitate greater understanding of the mechanisms of isolation and introgression. For species pairs such as the 2 trout species here, which have long been in sympatry, a combination of isolation mechanisms such as assortative mating, exogenous selection against less-fit hybrids, and intrinsic genomic incompatibilities may contribute to reduced fitness and asymmetric hybridization, and prevent the creation of a hybrid swarm. In contrast, in populations of cutthroat trout and rainbow trout/SH that are not historically sympatric, the outcome of introductions is often complete loss of the 2 parental types through introgressive hybridization (Allendorf and Leary 1988). Ultimately, understanding the geographic and genomic locations where hybridization occurs and the patterns within populations is critical to the conservation of cutthroat trout and rainbow trout/SH throughout their range.

Supplementary material

Supplementary material is available at *Journal of Heredity* online.

Supplementary Fig. S1. Position of landmarks used in distinguishing body shapes of CCT, SH, and their hybrids by analysis of geometric morphometrics. Illustration by Michael Zontos.

Supplementary Fig. S2. Stacked length histogram of sampled fish by hybrid class. Abbreviations for hybrid classes are defined in the text.

Supplementary Fig. S3. A) Mean Procrustes superimposition of large trout (FL > 200 mm) from the Smith River (CCT, n = 221; SH, n = 48). B) Mean procrustes superimposition of body shapes of small trout (FL ≤ 200 mm) from the Smith River (CCT, n = 148; SH, n = 131). Points represent the mean procrustes coordinates for each of 14 morphological landmarks. Vertical and horizontal bars represent one standard deviation around the mean. Orange circles (SH) and light blue circles (CCT).

Supplementary Fig. S4. FAMD scores for pairs of the first three axes, representing 57% of the variation in body shape of small individuals (FL ≤ 200 mm). Hybrid class, as defined by the NewHybrids analysis, is indicated by the circles for the parental types and triangles for the hybrid individuals. The morphometric residuals and phenotype scores from slash intensity and presence/absence of hyoid teeth were used as inputs for the FAMD. Abbreviations for hybrid classes are defined in the text.

Supplementary Fig. S5. FAMD scores for pairs of the first 3 axes, representing 57% of the variation in body shape of large individuals (FL > 200 mm). Hybrid class, as defined by the NewHybrids analysis, is indicated by the circles for the parental types and triangles for the hybrid individuals. The morphometric residuals and phenotype scores from slash intensity and presence/absence of hyoid teeth were used as inputs for the FAMD. Abbreviations for hybrid classes are defined in the text.

Supplementary Fig. S6. Individual genotypes (n = 876) at the 96 loci in order along the chromosomes. Light blue blocks represent homozygous CCT genotypes, light orange blocks represent homozygous SH genotypes, and brown blocks are heterozygous genotypes. Missing data (<1%), are shown in gray. Locus labels were made bold if they were used in the 30-marker hybrid analysis or italicized if they were not used in further analysis.

Supplementary Table S1. Hybrid classes included as possible categories for inference with NewHybrids and expected frequencies of the homozygous CCT, heterozygous (CCT/SH or SH/CCT), and homozygous SH genotypes, for each. Hybrid classes are defined as follows: CCT: pure coastal cutthroat trout; SH: pure steelhead; F1: first generation hybrid; F2: second generation hybrid (F1xF1); BxC: cross between F1 and CCT; BxSH: cross between F1 and SH; BxC2: cross between BxC and CCT; BxSH2: cross between BxSH and SH; BxCxBxC2: cross between BxC and BxC2; and BxSHxBxSH2: cross between BxSH and BxSH2. The column G_CCT (G_SH) gives the minimum number of generations in the pedigree between a pure CCT (SH) ancestor and an individual of each hybrid class.

Supplementary Table S2. Count of individuals in each hybrid class by size class (≤200 and >200 mm), for each combination slash intensity and maxillary length. Jaw slash color intensity was scored as not present—0, faint—1, or bright—2; maxillary shorter than posterior margin of eye—0, extends to posterior margin of the eye—1, or beyond the posterior margin of the eye—2. Abbreviations for hybrid classes are defined in the text.

Supplementary Table S3. Count of individuals in each hybrid class by size class (≤200 and >200 mm), for each combination slash intensity, maxillary length, and hyoid teeth. Individuals unable to be assessed for hyoid teeth were not included in the table. Jaw slash color intensity was scored as not present—0, faint—1, or bright—2; maxillary shorter than posterior margin of eye—0, extends to posterior margin of the eye—1, or beyond the posterior margin of the eye—2;

absence of hyoid teeth—0 or presence—1. Abbreviations for hybrid classes are defined in the text.

Supplementary Table S4. All loci assayed, with the associated frequency of the CCT and SH alleles calculated independently for each of the parentals. Pure CCT and SH were determined using the 30 "diagnostic" loci in NewHybrids. Missing data is reported at each locus for each parental. The locus type column identifies the 65 diagnostic loci, as well as the 30 "diagnostic" loci located on distinct chromosomes or separate arms along the same chromosome that were used in the NewHybrids analysis.

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Data availability

We have deposited the primary data underlying these analyses as follows: https://doi.org/10.5061/dryad.4mw6m90fn

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