ASYMMETRIC INTROGRESSION BETWEEN COASTAL CUTTHROAT TROUT AND STEELHEAD: VARIABLE INTROGRESSION BY LINKAGE GROUP

By

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A Thesis Presented to
The Faculty of Humboldt State University
In Partial Fulfillment of the Requirements for the Degree
Master of Science in Natural Resources: Fisheries

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July 2015
ABSTRACT

ASYMMETRIC INTROGRESSION BETWEEN COASTAL CUTTHROAT TROUT AND STEELHEAD: VARIABLE INTROGRESSION BY LINKAGE GROUP

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I assessed introgression between sympatric Coastal Cutthroat Trout (*Oncorhynchus clarki clarki*, CCT) and steelhead (*Oncorhynchus mykiss irideus*, SH) from 7 sub-basins of the Smith River in northern California. Population, individual and genomic level introgression was determined using a panel of 65 diagnostic single nucleotide polymorphisms, of which 59 are mapped to 26 of 28 known linkage groups. Among hybrids, first-generation hybrids (F1s) were rare (2%) and the frequency of backcrosses was asymmetric, with backcrosses to SH infrequent (<1%), and backcrosses to CCT relatively common (17%). Mitochondrial DNA of 14 of 15 F1 hybrids was of steelhead origin, suggesting that hybridization was driven by sneak-mating of male CCT with female SH. Genomic clines analysis located nine loci across three known linkage groups that deviated from a neutral model of introgression. Genome-wide differential introgression was documented along with non-random patterns of introgression among linkage groups. These findings suggest genomic blocks are inherited in recent CCT and SH hybrids and subsequently undergo decay as repeated rounds of recombination break up linkage group associations. Chromosome rearrangements known to suppress recombination are suspected in CCT and SH hybrids, and may preserve genomic regions fundamental to isolation. Analysis of geometric morphometrics and phenotypic
characteristics revealed distinct CCT and SH morphologies. However, first-generation hybrids and backcrosses expressed non-distinct morphologies that overlapped with parental types, creating challenges in visual field identification.
I would like to thank my advisor Dr. Margaret Wilzbach for giving me the opportunity to further my education. Your guidance and belief in me helped make my experience as a graduate student thoroughly enjoyable.

The constant interest and knowledge of Dr. Andrew Kinziger helped focus this research in the right direction. Your enthusiasm sparked my fascination of genetics, for which I am forever grateful. I can no longer look at fisheries without wondering about the genetics. Strangely, I now look forward to a world where full genome scans reveal an unimaginable complexity of patterns and questions.

Dr. Walt Duffy and Dr. Bret Harvey thank you for your comments, knowledge, and assistance along the way. Leslie Farrar thanks for the great conversation and all your behind the scenes work in the CA Fisheries Coop.

Dr. Carlos Garza and his Molecular Ecology Team at the National Oceanic and Atmospheric Administration Southwest Fisheries Science Center in Santa Cruz performed the genotyping and assisted in the direction of the analysis. Thank you for the opportunity visit your lab and observe the genotyping process.

To my field partner Jeff Abrams (who may have caught more fish than me), you are as good as they get. Your drive to catch fish was unmatched and ability to adapt to an underwater nocturnal lifestyle was a real benefit to the project. Also, Jesse Wiesenfeld’s help in the genetics lab and hints on how to succeed as a graduate student were of great value.
Financial assistance from the Danielle Plum Zumbrun Memorial Scholarship was greatly appreciated.

Finally, I would like to thank the California Department of Fish and Wildlife Heritage and Wild Trout Program for funding this research, which is unparalleled in its scope. I applaud Jeff Weaver and Roger Bloom’s passion for wild Coastal Cutthroat Trout and steelhead, and commitment to outfit us with the best gear available to perform this research.
TABLE OF CONTENTS

ABSTRACT ........................................................................................................................ ii

ACKNOWLEDGEMENTS ............................................................................................... iv

LIST OF TABLES ........................................................................................................... viii

LIST OF FIGURES ........................................................................................................ ix

LIST OF APPENDICES .................................................................................................. xi

INTRODUCTION ............................................................................................................. 1

METHODS ......................................................................................................................... 9
  Field Collections ........................................................................................................... 9
  Study Site .................................................................................................................... 9
  Field Collections ....................................................................................................... 11

Molecular Methods ...................................................................................................... 13
  Genetic Analysis ........................................................................................................ 14
  Morphometric and Phenotypic Analyses .................................................................. 16

RESULTS ......................................................................................................................... 20
  SNP Genotyping ....................................................................................................... 20
    Hybridization and Introgression ............................................................................. 20
  Morphometrics and Phenotype Classification .......................................................... 28
    Phenotypic Classification ....................................................................................... 34
    Field Identification ................................................................................................. 34

DISCUSSION ................................................................................................................... 35
  Genomic Clines and Architecture ............................................................................. 35
  Hybridization and Asymmetric Introgression ........................................................... 40
LIST OF TABLES

Table 1. Markers distinguishing between CCT and SH that deviated (P ≤0.005) from the neutral introgression model in trout sampled in the Smith River (n = 876), with associated linkage group and direction of the genomic cline. Positive (+) and negative (-) selection is indicated for homozygous genotypes AA (Coastal Cutthroat Trout). No selection for homozygous genotypes aa (steelhead) was observed. Overdominance (+) and underdominance (-) are shown for heterozygous genotypes Aa (hybrid)........................ 25

Table 2. Comparison of group means over the first three principal component axes of the geometric morphometric analysis. Size class small includes fish < 200 mm fork length and large includes fish >200 mm fork length. CCT represents Coastal Cutthroat Trout, CCT-like represents Coastal Cutthroat Trout possessing at least one alternate allele, and SH represents steelhead. PC1, PC2, and PC3 represent each of the first three principal 31
LIST OF FIGURES

Figure 1. Sub-basins of the Smith River, California, which were sampled in 2013 for coastal cutthroat trout, steelhead, and their hybrids. Each sub-basin is a distinct color. Inset shows location of study site in California. ............................................................... 10

Figure 2. Position of landmarks used in distinguishing body shapes of cutthroat trout, steelhead, and their hybrids by analysis of geometric morphometrics. Image was reproduced from Trout Unlimited (Shick 2015). .............................................................. 17

Figure 3. Hybrid index scores of Coastal Cutthroat Trout, steelhead, and hybrid individuals sampled in 2013 within the Smith River, CA. Genetic groups: SH- steelhead, SH-like- represents steelhead possessing at least one alternate allele, F1-first-generation hybrid, F2-second-generation hybrid, CCT-like, represents Coastal Cutthroat Trout possessing at least one alternate allele, and CCT- Coastal Cutthroat Trout. .................... 21

Figure 4. Number of sampled hybrid individuals per sub-basin of the Smith River, by genotype class. SH-like- represents steelhead possessing at least one alternate allele, F1- represents first-generation hybrid, F2-represents second-generation hybrid, and CCT-like-represents Coastal Cutthroat Trout possessing at least one alternate allele. ............. 23

Figure 5. Number of sampled hybrid individuals less than or equal to 200mm fork length per sub-basin of the Smith River, by genotype class. SH-like- represents steelhead possessing at least one alternate allele, F1-represents first-generation hybrid, F2-represents second-generation hybrid, and CCT-like- represents Coastal Cutthroat Trout possessing at least one alternate allele. ............................................................................. 24

Figure 6. Individual genotypes at loci of the 65 diagnostic markers (n =876). Coastal Cutthroat Trout that are homozygous at a locus are colored with dark green, homozygous steelhead are colored light green, and heterozygous individuals are colored green. Asymmetric introgression of steelhead into Coastal Cutthroat Trout is visible as light green and green rectangles descending into the dark green Coastal Cutthroat Trout individuals. Asterisks (*) below the x-axis represent 9 loci with significant genomic clines. Alternating yellow and blue numbered bars distinguish linkage groups. Figure on right shows relationship of hybrid index value to genotypes in figure on left. Missing.. 26

Figure 7. Composite plot of all 65 genomic clines overlaid on the 95% confidence interval (green) under a model of neutral introgression. Each cline represents the probability of a genotype at a locus given the hybrid index score. The plot on the left represents the probability of an AA genotype as the hybrid index increases from 0.0 (Coastal Cutthroat Trout) to 1.0 (steelhead). The plot on the right represents the probability of an Aa (hybrid) genotype as the hybrid index increases. The six genomic
clines on both plots to the left and outside of the 95% confidence intervals are loci associated with linkage group eight. These six genomic clines show introgression of heterozygous genotypes at low hybrid index scores, signifying increased introgression of steelhead alleles at linkage group eight. ................................................................. 27

Figure 8. Procrustes superimposition of body shapes of small trout (fork length ≤ 200mm) sampled from the Smith River (n =290). Open circles represents an individual’s procrustes coordinates for each of 14 morphological landmarks. Overlapping yellow (CCT- Coastal Cutthroat Trout and CCT-like-represents Coastal Cutthroat Trout possessing at least one alternate allele), black (F1-represents first-generation hybrid and F2- represents second-generation hybrid), and blue (SH-steelhead and SH-like- represents steelhead possessing at least one alternate allele) point clouds are shown for each landmark. .......................................................................................................................... 29

Figure 9. Procrustes superimposition of adult trout (fork length > 200mm) sampled from the Smith River (n =275). Open circles represents an individual’s procrustes coordinates for each of the 14 landmarks. Overlapping yellow (CCT- Coastal Cutthroat Trout and CCT-like-represents Coastal Cutthroat Trout possessing at least one alternate allele), black (F1-represents first-generation hybrid and F2- represents second-generation hybrid), and blue (SH-steelhead and SH-like- represents steelhead possessing at least one alternate allele) point clouds are shown for each landmark............................................. 30

Figure 10. Principal component scores for the first three axes representing 61% of the variation in body shape of small individuals (fork length < 200 mm) among genetic groupings of CCT, SH, and their hybrids. Genetic groups: CCT- Coastal Cutthroat Trout, CCT-like, represents Coastal Cutthroat Trout possessing at least one alternate allele, F1-first-generation hybrid, F2-second-generation hybrid, SH-like- represents steelhead possessing at least one alternate allele, and SH- steelhead.............................................. 32

Figure 11. Principal component scores for the first three axes representing 66% of the variation in body shape of large individuals (fork length > 200 mm) among genetic groupings of CCT, SH, and their hybrids. Genetic groups: CCT- Coastal Cutthroat Trout, CCT-like, represents Coastal Cutthroat Trout possessing at least one alternate allele, F1-first-generation hybrid, SH-like- represents steelhead possessing at least one alternate allele, and SH- steelhead................................................................. 33
LIST OF APPENDICES

Appendix A. Table of 65 diagnostic single nucleotide polymorphisms (SNPs) used to assess introgression of CCT and SH in the Smith River Basin. All SNPs except Ocl_gshpx-357 (Campbell et al. 2012) were discovered by Pritchard et al. (2012)....... 59

Appendix B. Figures describe the differences in procrustes coordinates at the morphological landmarks between small (< 200 mm fork length) CCT and SH at the first three principal components. .............................................................................................. 60

Appendix C. Figures describe the differences in procrustes coordinates at the morphological landmarks between large (> 200 mm fork length) CCT and SH at the first three principal components. .............................................................................................. 61
INTRODUCTION

The characterization of species boundaries at population (Mayr 1942) and genomic (Wu 2001) levels has shaped our perception of a species. Mayr (1942) formulated the Biological Species Concept, envisioning species boundaries as characterized by complete reproductive isolation between populations at a genome-wide scale. Complete reproductive isolation eliminates the possibility of two groups to fuse, often through geographic isolation (Barton and Hewitt 1985). However, new species can arise from a single ancestral species in sympatry (Taugbøl et al. 2014). In fact, Darwin (1859) proposed a continuum between varieties and species in which species boundaries were driven by differential adaptation and sexual selection. A more recent “genic view” suggests that genomic regions of reproductive isolation on chromosomes can maintain divergence between species despite consistent gene flow across other regions of the genome (Wu 2001). Genome-wide differential introgression that allows or restricts the passage of certain genes between species has been illustrated in several instances supporting the idea of the genome acting as a semipermeable membrane to introgression (Nolte et al. 2009, Teeter et al. 2010, Parchman et al. 2013). With recognition that reproductive isolation is best explored at the level of genes, Rieseberg et al. (1999) and Gompert and Buerkle (2013) maintain that the location and ultimately the description of these genes or genomic regions will come to guide speciation research.
Natural hybrid zones, locations where genetically distinct populations meet, mate, and produce hybrids, represent a window to the process of incipient speciation (Barton and Hewitt 1985). The complexity of hybrid genotypes attained after thousands of generations of recombination in a natural hybrid zone is unparalleled and cannot be recreated by the artificial propagation of various hybrids (Rieseberg et al. 1999). Barriers to interspecific gene flow or introgression are representative of the natural population because the fitness of hybrid genotypes has been tested against natural conditions (Rieseberg et al. 1999).

Two methods have been used to quantify introgression levels and their variance across the genome in hybrid zones: 1) geographic clines track the movement of alleles across space and 2) genomic clines measure the movement of ancestry blocks along a genome-wide admixture gradient (Gompert et al. 2012). Understanding the genomic mechanisms of reproductive isolation and the potential consequences of introgression at a genomic scale will help to inform our perception of speciation (Gagnaire et al. 2011).

Hybrid zone isolation mechanisms are dependent on how they were initially formed: 1) through sympatric evolution, or 2) secondary contact after allopatric speciation (Abbott et al. 2013). However, species histories are often not as simple as sympatric or allopatric speciation, and numerous species are thought to have undergone alternating periods of isolation and gene flow (Harrison and Larson 2014). For instance, geologic barriers are suspected to be the catalysts for allopatric divergence of the inland sub-species of Cutthroat Trout and Rainbow Trout (O. mykiss) (Allendorf and Leary 1988). Mating of inland sub-species of Cutthroat Trout with introduced Rainbow Trout
has led to introgressive hybridization in many Cutthroat Trout populations, particularly within West-slope (\textit{O. clarki lewisi}) and Yellowstone Cutthroat Trout (\textit{O. clarki bouvieri}) (Allendorf and Leary 1988). In contrast, closely related forms of Coastal Cutthroat Trout (\textit{O. clarki clarki}) (CCT) and steelhead (\textit{O. mykiss irideus}, SH) are presumed, from natural distributions and a common life history of anadromy, to have shared a long evolutionary history of sympatry (Campton and Utter 1985). Numerous barriers to introgression may exist in species that evolved in sympatry, with prezygotic barriers (e.g. assortative mating and spatial isolation) usually playing a stronger role in maintaining species integrity than postzygotic barriers (e.g. sterility, inviability, or reduced fitness) (Coyne and Orr 1997). One form of postzygotic selection, exogenous selection, is mediated through the adaptation of parental types to specific habitats (Jiggins and Mallet 2000) and is thought to contribute to maintaining species boundaries in the CCT and SH hybrid zone (Campton and Utter 1985, Young et al. 2001, Ostberg et al. 2004).

Studies of natural hybridization between CCT and SH have documented varying results: hybrid swarms or the loss of pure genotypes from extensive backcrossing (Bettles et al. 2005), asymmetric introgression (Baumsteiger et al. 2005), and near complete reproductive isolation (Young et al. 2001), revealing that patterns of hybridization are variable across the range of the species. Reproductive isolation between CCT and SH through spatial and temporal partitioning, assortative mating, and reduced hybrid fitness are thought to maintain species boundaries (Campton and Utter 1985, Trotter 1989, Ostberg et al. 2004, Buehrens et al. 2013). Whereas, anthropogenic disturbances (e.g. logging, urbanization, and hatchery stocking) have been found to induce higher
hybridization rates (Docker et al. 2003, Bettles et al. 2005, Heath et al. 2010). Limited instances of hybrid swarms within the CCT and SH hybrid zone have been documented revealing the potential for a complete breakdown of isolation mechanisms (Bettles et al. 2005, Heath et al. 2010).

Rainbow Trout and Cutthroat Trout are thought to have diverged from a common ancestor 3 million years ago, and 1.5 million years later inland sub-species of Cutthroat Trout diverged from CCT (Wilson and Turner 2009), resulting in varying chromosome numbers between species and among sub-species (Thorgaard 1983). The number of chromosomes in CCT (2N=68) (Behnke 1970) is higher than that of Rainbow Trout (2N=58-64). Though distinct karyotypes exist, 104 chromosome arm numbers are consistent in all sub-species of Rainbow and Cutthroat Trout, enabling hybridization. A constant number of chromosome arms stems from Robertsonian translocations between metacentric and acrocentric chromosomes. Robertsonian translocations involve centric fusion or fission between chromosome arms without affecting chromosome number (Ostberg et al. 2013). Differences in chromosome number may cause chromosome rearrangements that are thought to reduce gene flow by suppressing recombination, which conserves large blocks of species-specific DNA (Ostberg et al. 2013). Therefore, large differences in chromosome number between CCT and SH may facilitate intrinsic genetic isolation through the suppression of recombination.

Analysis of allozyme and microsatellite markers have been used to assess the extent of hybridization between CCT and SH, within watersheds across their range (Allendorf and Leary 1988, Baumsteiger et al. 2005, Buehrens et al. 2013). These
techniques are being supplanted by analysis of single nucleotide polymorphisms (SNPs), which allow high throughput and exhibit low genotyping error (Garvin et al. 2010). A panel of diagnostic SNPs has been assembled to differentiate CCT, SH, and their hybrids and reveal introgression (Pritchard et al. 2012, Campbell et al. 2012). Diagnostic loci accurately measure varying levels of introgression and are able to detect unique genomic patterns of introgression when loci are sufficiently abundant and dispersed throughout the genome (Payseur 2010).

The application of diagnostic markers located throughout the genome allows for an analysis of introgression at the genomic scale, providing the ability to locate genomic regions or loci that may promote or limit introgression (Payseur 2010, Teeter et al. 2010). Location of these genomic regions, fundamental in isolation or adaptation, furthers research that attempts to link genes with ecological, evolutionary, and physiological processes (Rieseberg et al. 1999, Noor et al. 2001, Pearse et al. 2014). In addition, a marker set with coverage over numerous linkage groups aids in characterizing the genome-wide variability of introgression (Payseur 2010).

Though CCT and SH live in sympatry throughout most of the range of CCT from northern California to southern Alaska (Trotter 1989, Gerstung 1997), they occupy modestly different niches and express morphologies adapted to species-specific life histories (Hawkins and Quinn 1996). Coastal Cutthroat Trout typically spawn in upper reaches of headwater streams (Buehrens et al. 2013), migrating downstream afterwards to inhabit deep slow water where they exhibit a piscivorous feeding behavior (Trotter 1989). Steelhead trout spawn lower in the watershed than CCT (Buehrens et al. 2013) and prefer
faster water velocities to feed on invertebrate drift at the juvenile life stage (Bisson et al. 1988). Anadromous CCT and SH migrate toward the ocean after spending 2-3 years in freshwater. Habitat use in the marine environment rarely overlaps between the species as CCT typically make only short estuarine migrations (Trotter 1989, Moore et al. 2010) while SH are known to undertake long-distance ocean migrations (Withler 1966). Adult CCT mature at 30-48 cm fork length (Trotter 1989) much smaller than SH who return from the ocean mature at 36-80 cm fork length (Withler 1966).

Visible morphological differences that distinguish CCT from SH provide the potential of geometric morphometrics to aid in hybrid identification (Hawkins and Quinn 1996). The field of landmark-based geometric morphometrics has shown promise in its ability to discern species and population differences based on shape alone (Depecker et al. 2006, Kennedy et al. 2009, Stelkens et al. 2009, 2012). The application of geometric morphometrics combined with genetic hybrid inference has been shown to aid in species identification and help explain morphological expression of hybrid classes (e.g. first-generation hybrid (F1) and backcrosses) (Nolte and Sheets 2005, Kennedy et al. 2009).

Diverse habitat preferences and behaviors of CCT and SH require capture techniques that can be adapted to a variety of stream characteristics. For example, preference of CCT for deep pools during summer, particularly as they increase in size (Heggenes et al. 1991), makes capturing them difficult. Because of the difficulties in sampling deeper water habitat previous studies have used electrofishing and weirs to collect primarily juvenile and smolting CCT (Ostberg et al. 2004, Baumsteiger et al. 2005, Kennedy et al. 2009, Buehrens et al. 2013). In the Smith River, deep pools created
by bedrock and large boulders limit the effectiveness and feasibility of electrofishing. Alternatively, techniques such as angling (Seiler and Keeley 2009, Peterson 2011) and night netting with a dipnet (Tattam 2006) are proven methods for capturing both small and large CCT and SH.

Coastal Cutthroat Trout remain the least studied and understood of the salmonid fishes of the Pacific Northwest, and the information needed to assess the status and trends of CCT populations is limited at both local and range-wide scales. Coastal Cutthroat Trout are not commercially harvested, and much of the information used to manage this species is gathered incidentally while sampling for other Pacific salmonid species, including Chinook Salmon (*Oncorhynchus tshawytscha*), Coho Salmon (*O. kisutch*), and SH. Morphological and physical characteristics that distinguish CCT and SH have been found to be less apparent at the juvenile life stage, compounding the cryptic nature of hybrids and the ability to accurately characterize abundance estimates where hybridization occurs (Baumsteiger et al. 2005, Buehrens et al. 2013). Wild SH are classified as threatened over most of their overlapping range with CCT and therefore provided with special angling protections. Hybrids of CCT and SH present a distinct management challenge due to their cryptic characteristics and ambiguous regulation status. Understanding the mechanisms of hybridization between CCT and SH is important to the management of these species.

The objectives of this study were to: 1) determine the extent of hybridization of CCT and SH in the Smith River Basin, 2) locate and assess the contribution of specific genomic regions to reproductive isolation and gene flow, 3) determine the frequency and
degree of introgression, and 4) characterize morphological expression over the range of introgression between CCT and SH.
METHODS

Field Collections

Study Site

The Smith River enters the Pacific Ocean in northwestern California close to the Oregon border. The river drains a 1,950 km$^2$ basin of steep forested terrain in the western Klamath and Siskiyou Mountains, the majority (87%) of which is in public ownership. Designated as a National Wild and Scenic River, the Smith is the largest free-flowing river in California and is renowned for its water clarity. Annual discharge near Crescent City averages 106 m$^3$/s, and is highly variable seasonally. The regional climate is characterized as marine west coast. The majority of precipitation falls as rain during winter, with annual amounts ranging from 190 cm along the coast to 320 cm at higher elevations (Iwatsubo and Washabaugh 1982).

The Smith River is considered a stronghold in California for cutthroat trout, steelhead, and salmon (Chinook salmon and Coho Salmon). Gerstung (1997) noted that the river provides 395 km of habitat for cutthroat trout, more than double the amount of any other California river. For this study, I divided the river into seven sub-basins: 1) the estuary (the mouth of the Smith River to Morrison Slough), 2) Rowdy Creek, 3) the main stem 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 (Figure 1). Landholdings
surrounding the estuary and in the western portions of the basin are mainly in private ownership, and are managed for agriculture and timber production.

Figure 1. Sub-basins of the Smith River, California, which were sampled in 2013 for coastal cutthroat trout, steelhead, and their hybrids. Each sub-basin is a distinct color. Inset shows location of study site in California.
Field Collections

I sought sample sizes of 130 trout (CCT and HY, n=100; SH, n=30) per sub-basin. For each sub-basin 100 putative CCT and HY, with fork lengths > 100 mm, were collected if they met any of the following criteria: red-orange coloration under jaw (jaw slashes), maxillary extension beyond the posterior margin of eye, or presence of basal branchial (hyoid) teeth. Thirty putative SH with a fork length > 100 mm were collected under the criteria: absence of red-orange coloration under the jaw and basal branchial teeth. I sought to collect more CCT than SH because an assessment of fine-scale population structure within CCT was an ancillary goal of this research. Targeting post-0-age fish (fork length > 100mm) lowered the probability of capturing closely related individuals by allowing sufficient mixing of siblings (Buehrens et al. 2013).

Subsampling within sub-basins partitioned samples sizes between the main stem and major tributaries while providing coverage throughout each sub-basin. In the South Fork Smith River sub-basin, sampling effort was divided among the main stem of the South Fork Smith River, Goose Creek, and Hurdygurdy Creek. Effort in the Middle Fork Smith River sub-basin was partitioned among the main stem of the Middle Fork Smith River, Patrick Creek, and the Siskiyou Fork. An additional set of 30 putative CCT was collected from a barrier-isolated section of Little Jones Creek in the Middle Fork Smith River sub-basin. In the North Fork Smith River, only California waters were sampled, providing samples from both the main stem of the North Fork Smith River and Diamond Creek. Mill Creek CCT sampling was divided between the East Fork and West Branch,
with the majority of the SH samples coming from the main stem of Mill Creek. Many of the fish captured from Mill Creek were collected at weirs operated by the California Department of Fish and Wildlife on the East Fork and West Branch during spring out-migration of salmonids from March through June.

Samples were collected mid-May 2013 through August 2013. Fish sampling and handling procedures were approved under Humboldt State University Institutional Animal Care and Use permit 12/13.F.77.A. Fish were captured using night netting, hook and line, electrofishing, and weirs. Hook and line sampling was effective during late spring, when CCT were feeding on newly hatched and smolting Chinook Salmon, Coho Salmon, and SH. Artificial lures imitating juvenile salmonids, with a large gap and a single barbless hook, were used to minimize injuries to the fish. As spring flows receded and water temperature and clarity increased, catch rate declined, and sampling by night netting of fish was most effective. Night netting involved temporarily blinding the fish with an underwater light-emitting diode light, and slowly working a net on either side or under the fish to capture the individual. Night netting did not appear to result in capture injuries. In addition, water temperatures were lower at night and fish were docile and much easier to handle.

Fish were anesthetized using Alka Seltzer Gold (0.5 tablet per 5 l of water) before measuring fork length (±1 mm) and mass (±1 g), and removing a tissue sample. Tissue samples ≤ 0.5 cm² were collected from the upper lobe of the caudal fin of each fish. Individuals were field-identified on the basis of phenotypic characteristics: jaw slash intensity, maxillary length, and presence of hyoid teeth. Phenotypic characteristics were
ranked for each individual as follows: jaw slash intensity (0= absent, 1=faint/incomplete, or 2=bright/full), maxillary length (0=ends before posterior margin of eye, 1=ends at posterior margin of eye, or 2=extends past posterior margin of eye), and hyoid teeth (0=absent, 1=present). A digital photo with a scale bar included was taken of each fish for geometric morphometric analysis.

Molecular Methods

DNA extraction from tissue samples followed Qiagen DNeasy protocol. Single nucleotide polymorphism genotypes were generated following the protocol for TaqMan assays, on 96.96 Dynamic Genotyping Arrays, with a pre-amplification step following the manufacturer’s protocol (Fluidigm Corporation). Single nucleotide polymorphism calls were manually scored using the SNP Genotyping Analysis Software (v 3.0.2, Fluidigm). A SNP panel was assembled from Pritchard et al. (2012), with an additional marker from Campbell et al (2012), consisting of 68 putatively diagnostic loci (fixed or nearly fixed for alternate alleles) between CCT and SH, based on previous validation studies across the range of CCT and SH (Pritchard et al. 2012). Loci were considered diagnostic if > 98% of the species-specific alleles were called in both the CCT and northern SH validation sets. The southern SH validation set was outside the current range of CCT, therefore loci polymorphic in southern SH were not considered diagnostic due to the inability to disentangle recent introgression and ancestral polymorphism. Putative diagnostic marker Ocl105714D was polymorphic within CCT of the Smith River and was removed from further analysis. The markers Ocl106479D and Ocl118654D
were removed as they suggested SH introgression into a putatively pure population of CCT in Little Jones Creek, potentially due to ancestral polymorphisms. The removal of the three suspected non-diagnostic markers left a panel of 65 diagnostic loci, which are listed in Appendix A. DNA was extracted and genotyped by the Molecular Ecology Team at the NOAA Southwest Fisheries Science Center, under the supervision of Dr. Carlos Garza.

A linkage map created for Yellowstone Cutthroat and Rainbow Trout hybrids by Ostberg et al. (2013) was used to determine whether patterns of introgression between CCT and SH were present within or between linkage groups. Due to the close relation of Yellowstone Cutthroat Trout with CCT and Rainbow Trout with SH the linkage map provides a general model of genomic introgression that is likely relevant (Ostberg et al. 2013). Sixty of the diagnostic loci were mapped to specific linkage groups in hybrids of Yellowstone cutthroat trout and rainbow trout. The mapped loci were widely distributed across the genome, occurring within 25 of 28 linkage groups, dependent on number of centric fusions or fission rearrangements in CCT and SH hybrids.

I assayed the mitochondrial gene, NADH dehydrogenase-1 (ND-1), in the Fisheries Genetics Laboratory at Humboldt State University to identify the maternal lineage (SH or CCT) of F1 hybrids (n =15). Mitochondrial haplotypes indicative of SH and CCT were identified following procedures described in Baumsteiger et al. (2005).

Genetic Analysis
Conformance of populations to Hardy-Weinberg equilibrium and tests for linkage disequilibrium, to ascertain whether genotypes at a given locus were independent from genotypes at all other loci, were conducted using the software Genepop 4.2 (Raymond and Rousset 1995). Fish from each of the seven sub-basins plus the barrier-isolated population in Little Jones Creek were treated as separate populations. Individuals were removed from analyses if genotypes were missing at >20% of the loci (n = 8 individuals). Remaining individuals (n=876) were included in all genetic analyses.

A hybrid index was estimated for each individual using the R package: Introgress (Gompert and Buerkle 2009). Hybrid index scores represent the proportion of alleles from one parental type, therefore scores ranged from 0 to 1, with 0 equal to pure CCT, and 1 equal to pure SH, and an approximate score of 0.5 representing F1 or second-generation hybrid (F2) individuals. Expectations of parental genotypes under diagnostic loci were used to differentiate between F1 and F2 hybrids, where F1 hybrids exhibit zero parental genotypes and F2 hybrids exhibit roughly 25% of each parental genotype. The terms “CCT-like” and “SH-like” were used to designate later generation hybrid individuals backcrossed toward a particular parental type.

A genomic clines analysis, to test for loci that deviated from a neutral model of introgression, was conducted using the R package Introgress (Gompert and Buerkle 2009). Multinomial regression was used to estimate individual-based clines of genotype frequency across an admixture gradient (hybrid index) for each locus. The observed genotypes were permutated (n=1000) across the loci to form a neutral model of introgression under the assumption that loci are exchangeable. Estimated clines were
tested against the neutral model of introgression, with significant deviations representing loci potentially under selection due to adaptation or reproductive isolation. The shape of the estimated genomic clines compared to the neutral model allows for characterization of loci as overdominant (increased selection) or underdominant (decreased selection) for heterozygous genotypes, and for detection of positive or negative selection of homozygous genotypes. To correct for multiple tests of significance I applied the false discovery rate procedure (Benjamini and Hochberg 1995).

Morphometric and Phenotypic Analyses

I compared body shapes of CCT, SH, and their hybrids using landmark-based geometric morphometrics. Unlike traditional morphometrics, which uses angles and distances between morphological attributes to compare individuals, 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 loci that are homologous in all individuals, were superimposed 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). I chose landmarks known to be repeatable that together provided a complete description of body shape (Zelditch et al. 2012). These included: 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 (Figure 2). To ensure landmark placement did not migrate and to adjust for imperfect digitizing, each fish was measured twice and results of digitizing events were averaged (Klingenberg 2011).

Figure 2. Position of landmarks used in distinguishing body shapes of cutthroat trout, steelhead, and their hybrids by analysis of geometric morphometrics. Image was reproduced from Trout Unlimited (Shick 2015).

Sampled individuals were not included in the geometric morphometric analysis if the image quality was poor (e.g. photo was not taken from directly above the fish, the mouth of the fish was open, image quality was poor, or the caudal peduncle was not in line with the rest of the body), or if the genetic identity of the individual could not be established.
(e.g. genotypes were missing at greater than 20% of the loci). Of 886 fish sampled, 565 individuals had an accompanying photo that was used in the geometric morphometric analysis. The program tpsDIG2 (Rohlf 2013) was used to digitize the photographs. A scale factor was calculated for each photo by measuring a known distance on the photo (usually 150 mm, the length of the scale bar included in each photo). The known distance of the scale bar was converted into pixels and a scale factor was calculated to account for differences in focal length among photos.

Morphometrics were analyzed using program Morpho J (Klingenberg 2011). Because morphology varies with ontogeny (Klingenberg 1998) I separately analyzed fish less than or equal to and greater than 200 mm fork length. The size cutoff placed at 200 mm: 1) is approximately the size when CCT undergo smoltification (Trotter 1989), a process with the potential to influence body morphology and 2) limits sample size issues that arise from splitting the dataset. I applied a procrustes superimposition to minimize the sum of squared distances between landmarks (Klingenberg 2011). 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 (Klingenberg 2011). 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, or differential growth rates within a living organism. Residuals were used in a principal components analysis to identify the major axes of shape variation, and as an ordination method to view shape variation between genotype groups (Klingenberg 2011). Scores from the first three principal components were
plotted to visually inspect shape differences among genetic groups (i.e. CCT, CCT-like, F1, F2, SH-like, and SH) for both the small and large size classes separately.

Differences in phenotype patterns among genetic groups were analyzed using classification tables of each phenotypic characteristic (i.e. maxillary length, slash intensity, and presence or absence of hyoid teeth). Effect of body length on phenotypic characteristics was assessed by separately analyzing individuals using the small and large size classes.
RESULTS

SNP Genotyping

Hybridization and Introgression

The application of this marker panel in the Smith River Basin hybrid zone revealed pure populations of CCT and SH with limited hybridization. All populations and diagnostic loci failed to conform to Hardy-Weinberg equilibrium ($P<0.01$) and were under linkage disequilibrium ($P<0.01$).

Hybrids, individuals possessing at least one alternate allele over the 65 diagnostic loci, comprised 19% (168 of 876) of the fish sampled. The presence of SH alleles in CCT-like individuals ($n = 135$ individuals) was more commonly observed than was the presence of CCT in SH-like individuals ($n = 2$) (Figure 3), suggestive of asymmetric introgression. Analysis of F1 mtDNA revealed a strong pattern of asymmetric mating of SH females and CCT males. Fourteen of 15 (93.3%) F1 hybrids had mtDNA from SH.
Figure 3. Hybrid index scores of Coastal Cutthroat Trout, steelhead, and hybrid individuals sampled in 2013 within the Smith River, CA. Genetic groups: SH- steelhead, SH-like- represents steelhead possessing at least one alternate allele, F1-first-generation hybrid, F2-second-generation hybrid, CCT-like, represents Coastal Cutthroat Trout possessing at least one alternate allele, and CCT- Coastal Cutthroat Trout.

Differences in incidence of hybridization among sub-basins were not apparent. Hybrid individuals of at least one class (i.e. CCT-like, F1, F2, or SH-like) were found
within all seven sub-basins (Figure 4). All individuals sampled from Little Jones Creek were determined to be pure CCT. The majority of individuals identified as F1 hybrids (11 of 15) were captured from the estuary and from Rowdy Creek. Three F1 individuals, with a fork length ≤ 200 mm, were sampled in the Middle and South Fork Smith River. Among individuals ≤ 200 mm, at least one hybrid class was represented in all of the tributaries to the main stem of the Smith River (Figure 5). These individuals were assumed to represent stream-of-origin fish, as 200 mm is often a size regarded as too small to make long upstream migrations (Arnold 1974).
Figure 4. Number of sampled hybrid individuals per sub-basin of the Smith River, by genotype class. SH-like- represents steelhead possessing at least one alternate allele, F1- represents first-generation hybrid, F2- represents second-generation hybrid, and CCT-like-represents Coastal Cutthroat Trout possessing at least one alternate allele.
Figure 5. Number of sampled hybrid individuals less than or equal to 200mm fork length per sub-basin of the Smith River, by genotype class. SH-like- represents steelhead possessing at least one alternate allele, F1-represents first-generation hybrid, F2-represents second-generation hybrid, and CCT-like- represents Coastal Cutthroat Trout possessing at least one alternate allele.

Genomic Clines

While introgression at most loci conformed to a model of neutral introgression, 9 loci (14%), from 3 known linkage groups, had genomic clines that deviated from a
neutral model of introgression (P ≤ 0.007, Table 1). Deviations from neutrality consisted of loci with positive (n=2) and negative (n=6) selection of homozygous CCT genotypes.

Loci that shared linkage groups typically had similar patterns of introgression (Figure 6). For example, linkage group 8 consisted of nine loci, all with similar patterns of decreased selection of homozygous CCT genotypes and introgression at low hybrid index scores (Figure 7). Linkage group 5 consisted of three loci with similar patterns of increased selection of homozygous CCT genotypes and reduced introgression of alternate alleles.

Table 1. Markers distinguishing between CCT and SH that deviated (P ≤ 0.007) from the neutral introgression model in trout sampled in the Smith River (n = 876), with associated linkage group and direction of the genomic cline. Positive (+) and negative (-) selection is indicated for homozygous genotypes AA (Coastal Cutthroat Trout). No selection for homozygous genotypes aa (steelhead) was observed. Overdominance (+) and underdominance (-) are shown for heterozygous genotypes Aa (hybrid).

<table>
<thead>
<tr>
<th>Marker</th>
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<th>Linkage Group</th>
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<th>Aa</th>
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Figure 6. Individual genotypes at loci of the 65 diagnostic markers (n = 876). Coastal Cutthroat Trout that are homozygous at a locus are colored with dark green, homozygous steelhead are colored light green, and heterozygous individuals are colored green. Asymmetric introgression of steelhead into Coastal Cutthroat Trout is visible as light green and green rectangles descending into the dark green Coastal Cutthroat Trout individuals. Asterisks (*) below the x-axis represent 9 loci with significant genomic clines. Alternating yellow and blue numbered bars distinguish linkage groups. Figure on right shows relationship of hybrid index value to genotypes in figure on left. Missing
data (1%), replaced with dominant genome-wide genotypic expression for ease of viewing, were not used in statistical analysis.

Figure 7. Composite plot of all 65 genomic clines overlaid on the 95% confidence interval (green) under a model of neutral introgression. Each cline represents the probability of a genotype at a locus given the hybrid index score. The plot on the left represents the probability of an AA genotype as the hybrid index increases from 0.0 (Coastal Cutthroat Trout) to 1.0 (steelhead). The plot on the right represents the probability of an Aa (hybrid) genotype as the hybrid index increases. The six genomic clines on both plots to the left and outside of the 95% confidence intervals are loci associated with linkage group eight. These six genomic clines show introgression of heterozygous genotypes at low hybrid index scores, signifying increased introgression of steelhead alleles at linkage group eight.
Morphometrics and Phenotype Classification

Differences in morphology between CCT and SH existed primarily with respect to body depth, snout length, and eye location and size as shown by the procrustes coordinates (Figure 8 and 9). Shape differences between small (≤ 200 mm fork length) CCT and CCT-like individuals were not detected over the first three principal components axes, nor were shape differences detected between large (> 200 mm fork length) CCT and CCT-like individuals (Table 2) (Figures 10 and 11). Small CCT and SH occupied distinct morphospaces with significantly different group means at each of the first three principal component axes. The group means of the large CCT and SH were significantly different at each of the first three principal components, but a distinct separation between the species was not visible over the morphospace (Table 2) (Figures 10 and 11). First-generation hybrids occupied a morphospace that overlapped both CCT and SH, thus F1 were unable to be distinguished by geometric morphometrics. Shape changes over each of the first three principal component axes reflected the major differences shown by the plots of the procrustes coordinates (Appendix B).
Figure 8. Procrustes superimposition of body shapes of small trout (fork length ≤ 200mm) sampled from the Smith River (n =290). Open circles represents an individual’s procrustes coordinates for each of 14 morphological landmarks. Overlapping yellow (CCT- Coastal Cutthroat Trout and CCT-like-represents Coastal Cutthroat Trout possessing at least one alternate allele), black (F1-represents first-generation hybrid and F2- represents second-generation hybrid), and blue (SH-steelhead and SH-like- represents steelhead possessing at least one alternate allele) point clouds are shown for each landmark.
Figure 9. Procrustes superimposition of adult trout (fork length > 200mm) sampled from the Smith River (n =275). Open circles represents an individual’s procrustes coordinates for each of the 14 landmarks. Overlapping yellow (CCT- Coastal Cutthroat Trout and CCT-like-represents Coastal Cutthroat Trout possessing at least one alternate allele), black (F1-represents first-generation hybrid and F2- represents second-generation hybrid), and blue (SH-steelhead and SH-like- represents steelhead possessing at least one alternate allele) point clouds are shown for each landmark.
Table 2. Comparison of group means over the first three principal component axes of the geometric morphometric analysis. Size class small includes fish ≤ 200 mm fork length and large includes fish >200 mm fork length. CCT represents Coastal Cutthroat Trout, CCT-like represents Coastal Cutthroat Trout possessing at least one alternate allele, and SH represents steelhead. PC1, PC2, and PC3 represent each of the first three principal components of the geometric morphometric analysis. The t-value is represented by t and degrees of freedom by df.

<table>
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Figure 10. Principal component scores for the first three axes representing 61% of the variation in body shape of small individuals (fork length $\leq$ 200 mm) among genetic groupings of CCT, SH, and their hybrids. Genetic groups: CCT- Coastal Cutthroat Trout, CCT-like, represents Coastal Cutthroat Trout possessing at least one alternate allele, F1-first-generation hybrid, F2-second-generation hybrid, SH-like- represents steelhead possessing at least one alternate allele, and SH- steelhead.
Figure 11. Principal component scores for the first three axes representing 66% of the variation in body shape of large individuals (fork length > 200 mm) among genetic groupings of CCT, SH, and their hybrids. Genetic groups: CCT- Coastal Cutthroat Trout, CCT-like, represents Coastal Cutthroat Trout possessing at least one alternate allele, F1-first-generation hybrid, SH-like- represents steelhead possessing at least one alternate allele, and SH- steelhead.
Phenotypic Classification

The ability to distinguish phenotypic traits between CCT and SH differed between the small and large size classes. For example, 21 of 22 (95%) SH individuals with a maxillary that extended beyond the posterior margin of the eye (typical of CCT) belonged to the large size class. Sixty-six of 77 (87%) CCT and CCT-like individuals without the red-orange slash coloration under jaw, which usually characterizes cutthroat trout, belonged to the large size class. Notably, among small fish, 150 of 151 SH and SH-like individuals could not be scored for presence/absence of hyoid teeth because their mouths were too small.

Field Identification

Based on genotypic determination of species identity, 80 % of field-identified hybrids were correctly assigned. The majority of incorrect field assignments came from misidentification of backcrossed individuals. Seven of 15 F1 individuals were correctly identified in the field, and the F2 individual was incorrectly classified as a CCT.
DISCUSSION

Previous studies have pointed to extrinsic ecological and behavioral mechanisms in the maintenance of species boundaries of sympatric CCT and SH, through spatial and temporal partitioning of resources and divergent life histories of CCT and SH (Campton and Utter 1985, Ostberg et al. 2004, Buehrens et al. 2013). My research supports a conclusion that species boundaries are largely intact, and suggests that intrinsic genomic incompatibilities may also play a role in reproductive isolation mechanisms. The genomic clines analysis highlighted a number of loci and regions of the hybrid genome that deviated from the genome-wide expectation of neutral introgression. Genome-wide variable introgression across loci was present, but non-random patterns within linkage groups suggest regions of isolation or gene flow that may be targets of selection.

Genomic Clines and Architecture

The pattern of genome-wide variable introgression has been previously observed in hybrid zones (e.g., Payseur 2010, Teeter et al. 2010, Hermansen et al. 2014). Determining the genomic architecture or composition of these variable regions, however, is more challenging. Two opposing theories have been posited to account for genomic reproductive isolation: 1) as genomic islands, or discrete blocks of loci responsible for reproductive isolation or adaptation (Hoffmann and Rieseberg 2008, Noor and Bennett 2009); or 2) as loci scattered throughout the genome involved in reproductive isolation and adaptation (Feder et al. 2012, Parchman et al. 2013, Gompert and Buerkle 2013).
The genomic island theory has been criticized as a theory without quantitative basis, except in the case of chromosome rearrangements (Nosil and Feder 2012). Chromosome rearrangements have been found to suppress recombination, allowing the inheritance of blocks of loci that may contribute to reproductive isolation (Noor and Bennett 2009). Ostberg et al. (2013) found two confirmed chromosome rearrangements in hybrids of Yellowstone Cutthroat Trout and Rainbow Trout, with suppressed recombination. Ostberg suggested a greater difference in chromosome number between CCT and SH compared to Yellowstone Cutthroat Trout and Rainbow Trout would increase the number of chromosome rearrangements (personal communication, January 15, 2015). Given the potential for chromosome rearrangements to suppress recombination, support exists in the CCT and SH hybrid zone for genomic islands as building blocks in the architecture of reproductive isolation.

Other studies suggest that genome-wide scatterings of loci are responsible for reproductive isolation in hybridizing species. For example, Parchman et al. (2013) found many loci of varying effects represented by small ancestral blocks due to repeated recombination in the hybrid zone of Central American Manakins, *Manacus candei* and *M. vitellinus*. The Smith River Basin CCT and SH hybrid zone exhibited similar population level characteristics of introgression to that of the *M. candei and vitellinus* hybrid zone, characterized by asymmetric introgression, a low frequency of F1 hybrids, and a majority of introgression in the form of distant backcrosses. Genome-wide variable introgression in the CCT and SH hybrid zone was predominantly observed in the more distant backcrosses, suggesting a similar decay of linkage group size as recombination.
breaks down loci associations. The 59,100 SNPs used in *M. candei and vitellinus* hybrid zone were insufficient to characterize a recombination history or locate quantitative trait loci associations, highlighting both the complexity recombination can create and high level of genomic coverage needed to address these questions.

Differences in chromosome number between CCT and SH, and known chromosome rearrangements in hybrid sub-species point toward a genomic architecture containing genomic islands. However, Ostberg et al. (2013) found evidence of chromosome rearrangements and suppressed recombination in F1 hybrids, a genetic group most susceptible to chromosome rearrangements and likely to inherit large ancestral genomic blocks (Gompert and Buerkle 2013). My findings show similar patterns of introgression within linkage groups for recent CCT-like individuals, and a rapid decay of linkage group structure as CCT-like become more like the parental type. These patterns suggest that genomic blocks in CCT and SH hybrids are inherited in recent hybrids and subsequently broken down by recombination in later backcrossing events, resulting in a genome-wide scatter of variable introgression. Although the size of these blocks cannot be determined due to the unknown locations of loci within linkage groups and low marker density, the non-random patterns of introgression by linkage group in recent hybrids cannot be ignored. Gompert and Buerkle (2013) noted that genetic ancestry and allelic state are not equivalent, and that two identical nucleotides or chromosome segments may arise from different genetic ancestries (Gompert and Buerkle 2013). Complex genetic ancestries of varying chromosome number may be generated through the repeated backcrossing of hybrids with CCT and SH, creating further
challenges in understanding reproductive isolation. Future research in genetic mapping of hybrid genomes and applications of high marker density are needed to further elucidate the genomic architecture of CCT and SH hybrids.

In the face of ongoing hybridization, genomic regions of reproductive isolation may be important to maintaining species boundaries should prezygotic mechanisms fail to prevent gene flow. The semipermeable nature of the CCT and SH hybrid genome is best shown in the loci representing linkage groups 7-9. Near complete barriers to introgression are present in loci on linkage groups 7 and 9; introgression was not observed in distant backcrosses. However, the genomic clines and patterns of introgression at linkage group 8 suggest that selection of hybrid genotypes occurs in the region. The patterns of increased introgression seen at linkage group 8 suggest they are not the result of suppressed gene flow due to chromosome rearrangements, although they appear to introgress as a block in recent CCT-like individuals. Fitness related selection could cause this phenomenon, wherein alleles associated with fitness would drive selection toward the most fit species, in this case selection of SH alleles by CCT-like individuals (Barton and Hewitt 1985). My results suggest repeated rounds of recombination breaks down these fitness-linked associations in hybrid individuals, thus maintaining species boundaries.

Comparison of the same marker set across the CCT and SH range, from Alaska to northern California would have the potential to highlight loci or regions responsible for reproductive isolation, as all markers should show consistent reduced introgression (Nolte et al. 2009, Payseur 2010, Teeter et al. 2010). The variation in chromosome
number within SH populations (2N=58-61) observed along the Pacific Coast from Alaska to the Oregon border (Thorgaard 1983) could lead to fluctuations in introgression due to increased or decreased chromosome rearrangements. Moreover, multiple applications of the same marker set could help to differentiate between genetic drift and selection of specific traits (Rieseberg et al. 1999). Identification of loci responsible for reproductive isolation or local adaptation among hybrid zones is fundamental to the integration of phenotype and genotype (Payseur 2010). An ultimate goal is the identification of quantitative trait loci that are linked to or contain the genes which maintain species boundaries between CCT and SH.

Hybrid zone studies may unknowingly mischaracterize introgression when a small number of markers are analyzed or the same genomic region is repeatedly sampled (Payseur 2010). Because of unequal sampling of linkage groups within the genome I was not able to compare the number of loci expressing positive or negative selection of homozygous genotypes or models of overdominance or underdominance in heterozygous genotypes.

Payseur (2010) discussed the pitfalls and benefits to the exclusive use of diagnostic markers in hybrid zone studies. On one hand, diagnostic loci can underestimate the amount of introgression, which may be relevant in this study as four loci with potential ancestral polymorphisms in SH were not included in the analyses. These loci showed increased introgression of CCT alleles into the SH genome which would have resulted in more distant SH-like individuals. The continued absence of recent SH-like individuals would not have changed the overall pattern of population level
asymmetric introgression. Alternatively, including only diagnostic loci in comparisons between loci may increases the ability to locate loci of decreased introgression (Payseur 2010). This is especially applicable in the Smith River Basin CCT and SH hybrid zone where limited introgression exists.

Hybridization and Asymmetric Introgression

Analysis of linkage disequilibrium and Hardy-Weinberg equilibrium revealed a hybrid zone with limited interspecific mating and patterns of introgression characterized by diagnostic loci that were in linkage disequilibrium. Diagnostic loci often are in linkage disequilibrium, as they represent locations in the genome where consistent patterns of introgression are thought to be present (Goodman et al. 1999). Whereas Hardy-Weinberg equilibrium would typically only occur in the presence of a hybrid swarm as a consequence of non-selective mating between species (Bettles et al. 2005). The majority of the hybridization detected was extremely diluted by successive backcrossing (n=140 with < 6; n=30 with ≥ 6 invasive alleles) suggesting that selective forces act against recent hybrids, as only 3% of the sample represented F1, F2, or recent backcrossed individuals.

The direct cause of asymmetric gene flow is uncertain, as many factors including population structure, mating behavior, and fitness differences, are known to produce directional introgression (Barton and Hewitt 1985). Differences in population structure, for example, can result in increased interspecific mating at low densities of one or both species (Bettles et al. 2005). However, low or disproportionate relative densities of CCT
or SH were not in evidence during our spatially extensive sampling in the Smith River Basin. Mate choice may pose a barrier to introgression, particularly when run timing and spawning habitat of CCT and SH overlap (Buehrens et al. 2013). Highly skewed mtDNA of F1 hybrids toward SH and a low incidence of interspecific hybridization is consistent with findings of previous studies (Hawkins and Foote 1998, Ostberg et al. 2004, Baumsteiger et al. 2005) and suggests assortative mating between species. Ostberg et al. (2004) proposed two explanations for asymmetric SH mtDNA in F1 hybrids: 1) a male CCT sneak mating strategy, where a male CCT sneaks fertilizations from a female SH and 2) weakened assortative mating selection by female SH on the spawning grounds in the absence of a male SH, resulting in “settling” for a male CCT (Wirtz 1999).

Explanations for asymmetric mtDNA of F1 hybrids assume strong assortative mating between CCT and SH, with the potential to carry over to reinforcement in the form of mate selection by parental types against F1 hybrids. Expression of reinforcement through “behavioral sterility”, sexual selection against F1 hybrids, has been documented in hybrids zones of fly catchers (*Ficedula hypoleuca* and *F.albicollis*), wolf spiders (*Schizocosa ocreata* and *S. rovneri*) (Scheffer et al. 1996) and neotropical butterflies (*Anartia fatima* and *A. amathea*) (Devis et al. 1997), where phenotypic differences between hybrids and parental types are apparent. Geometric morphologies of F1 hybrids overlapped both of the parental types with 47% (7 of 15) of F1 hybrids correctly classified in the field, suggesting that F1 hybrids are occasionally morphologically distinguishable from parental types. Assuming the absence of selection, a hybrid population would exhibit first-generation backcrosses at twice the number of F1 (n=15)
individuals (Goodman et al. 1999). That no first-generation SH-like individuals were sampled while seven potential first generation CCT-like (hybrid index ≥ 0.25) were sampled suggests a potential SH bias against F1 hybrids. An obvious size difference in adult SH and CCT could lead to selection against the intermediate F1 hybrid by the larger SH. Further research is need to substantiate these findings, however numerous challenges exist in the observation of hybrid mating.

    Fitness differences between two species can also contribute to asymmetric gene flow. Hawkins and Foote (1998) found that egg size and development of CCT and SH offspring, spawned in a laboratory setting, differed depending on parental type of dam. Hybrid offspring with SH dams emerged earlier and had a larger yolk sack 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 hybrid mtDNA. A small number of F1 hybrids (n=15) ranging in length from153-401 mm (mean = 242 mm) suggest hybridization in the Smith River is perpetuated by limited but continual occurrence of interspecific mating.

    Morphological adaptations such as increased length and depth of adult SH (Withler 1966) compared to CCT (Trotter 1989) coincide with life history (e.g. ocean versus estuary migration) and habitat preferences (e.g. fast versus slow water). Hawkins and Quinn (1996) found intermediate hybrid individuals held a competitive advantage over CCT, a possible fitness related explanation for asymmetric introgression of SH alleles into CCT. However, decreased fitness of F1 hybrids and backcrossed SH
attempting taxing ocean migrations may lead to increased mortality resulting in a reduced presence of these hybrid types (Ostberg et al. 2004). Variation in adult migration patterns among CCT, SH, and their hybrids are not well studied. Moore et al. (2010) reported that acoustic tagged juvenile CCT, SH, and their hybrid displayed distinct migration patterns within Puget Sound, with hybrids traveling an intermediate distance, further than CCT but truncated in comparison to SH. The highly specialized morphologies and life histories of sympatric CCT and SH suggest exogenous selection against anadromous F1 hybrids and SH-like individuals maintains reproductive isolation. Most likely a combination of isolation mechanisms such as prezygotic assortative mating, exogenous selection against less fit hybrids attempting ocean migrations, and intrinsic genomic incompatibilities contribute to reproductive isolation and asymmetric introgression in the CCT and SH hybrid zone of the Smith River Basin.

While many studies have quantified hybridization between CCT and SH (Allendorf and Leary 1988, Young et al. 2001, Ostberg et al. 2004, Bettles et al. 2005, Baumsteiger et al. 2005, Kennedy et al. 2009, Buehrens et al. 2013), a common set of criteria for distinguishing hybrid or introgressed individuals has not been adopted. Several studies use allele counts, with individuals possessing at least one alternate allele being considered a hybrid. Allele counts are similar to the hybrid index used in this study, in that individuals with any amount of introgression were considered hybrid (Gompert and Buerkle 2009). Assays of differing sets and numbers of markers, combined with natural variation in genomic composition between populations, create challenges in quantifying and comparing hybridization among studies and locations.
High throughput SNP genotyping and the identification of CCT and SH diagnostic loci are becoming less expensive and more accessible, allowing increased genomic detail of hybridization and introgression (Hohenlohe et al. 2013, Gompert and Buerkle 2013, Baldassarre et al. 2014). These advances in genomic methods and technologies continue to provide insight into hybridization. Increased genome coverage may lead to functional explanations for introgression of species-specific traits under selection (Payseur 2010, Hohenlohe et al. 2013).

Coastal Cutthroat Trout and SH hybrids made up 19% of the sampled fish, which falls within the range reported in other CCT/SH hybridization studies 10-36% (California), 23% (Washington) (Buehrens et al. 2013), 35% (Washington) (Ostberg et al. 2004), and 21-88% (British Columbia) (Bettles et al. 2005). Estimates of the incidence of hybridization from this study should be considered with caution, as preset and unequal sampling between CCT/HY and SH (favoring CCT/HY to SH by 3:1), combined with a finding of asymmetric introgression favoring SH introgression into CCT, may bias the overall hybridization estimate (Allendorf et al. 2001). Disproportionate sample sizes of CCT and SH likely had little effect on the direction of asymmetric hybridization, as the signal was exceptionally strong.

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). In this study, sampling adult life stages of CCT and hybrids allowed a more complete life history to play out.
Though minimal introgression of CCT into SH was detected, further sampling of adult SH is needed to assess the occurrence of SH-like individuals at the adult life stage. Sampling of juveniles can lead to an increased sampling of siblings, which can also affect the incidence of hybrids (Hansen et al. 1997). Sampling throughout juvenile and adult life stages and accounting for over sampling siblings through limited localized sampling will allow for more accurate estimation of hybridization and introgression.

**Geometric Morphometrics and Phenotypic Characteristics**

My finding of a strong separation between CCT and SH morphologies, with F1 hybrids exhibiting non-distinct morphologies overlapping that of parental types is consistent with findings of Kennedy et al. (2009) from Abernathy Creek, Washington. The absence of distinctive morphologies prevented F1 hybrids from being readily distinguished in the field or through morphometric analysis. CCT-like and SH-like individuals occupied a morphospace that was similar to the dominant parental type resulting in zero correctly field-identified as hybrids. Advancements in genetic techniques, particularly an increase in number of loci assayed, have led to fine-scale analysis of introgression (Nolte et al. 2009, Parchman et al. 2013), in which genomic distinction may not translate into phenotypic expression (Allendorf and Leary 1988).

Phenotypic classification using the length of the maxillary, intensity red-orange jaw coloration, and presence or absence of hyoid teeth was unsuccessful to distinguish genetic groups. The separation of individuals into small and large size classes showed a reduced performance of phenotypic characteristics to classify CCT and SH dependent on
size class. Specifically, large SH expressed longer maxillaries, suggesting that maxillary length relative to the posterior margin of the eye is a characteristic only to be assessed on fish belonging to the small size class. Coastal Cutthroat Trout and CCT-like individuals in the large size class were less likely to exhibit red-orange jaw coloration. One potential explanation is the loss of coloration during smoltification (Johnson et al. 1999), a process that would occur in adult iteroparous CCT. Finally, the presence or absence of hyoid teeth was the most accurate phenotypic characteristic in distinguishing CCT from SH. However, an overwhelming majority of SH belonging to the small size class was unable to be scored due to decreased mouth size, limiting the use of hyoid teeth to identify small SH. My results demonstrate the inability to identify genetic classes from phenotypic characteristics.
LITERATURE CITED


Peterson, M. L. 2011. Possible decline in the half-pounder life history among Trinity River steelhead (*Oncorhynchus mykiss*). Humboldt State University.

Pritchard, V. L., A. Abadía-Cardoso, and J. C. Garza. 2012. Discovery and characterization of a large number of diagnostic markers to discriminate *Oncorhynchus mykiss* and *O. clarkii*. Molecular Ecology Resources 12:918–931.


APPENDIX A

Appendix A. Table of 65 diagnostic single nucleotide polymorphisms (SNPs) used to
assess introgression of CCT and SH in the Smith River Basin. All SNPs except
Ocl_gshpx-357 (Campbell et al. 2012) were discovered by Pritchard et al. (2012).

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Appendix B. Figures describe the differences in procrustes coordinates at the morphological landmarks between small (≤ 200 mm fork length) CCT and SH at the first three principal components.
Appendix C. Figures describe the differences in procrustes coordinates at the morphological landmarks between large (> 200 mm fork length) CCT and SH at the first three principal components.