Wild Fish Conservancy
NORTHWEST
SCIENCE EDUCATION ADVOCACY

FINAL REPORT
WNTI Agreement 2012SG-1
Upper Wynoochee Bull Trout Population Assessment

Background and Goals:
A 2008 report prepared for the U.S. Fish and Wildlife Service by the Bull Trout Recovery and Monitoring Technical Group (RMEG) states that "Bull trout (*Salvelinus confluentus*) is an imperiled species of char native to the Pacific Northwest. Combinations of habitat degradation (e.g., Fraley and Shepard 1989), barriers to migration (e.g., Rieman and McIntyre 1995), and the introduction of non-natives (e.g., Leary et al. 1993) have led to the decline of bull trout populations across their native range (Rieman et al. 1997). Consequently, bull trout in the coterminous United States were listed as threatened, under the Endangered Species Act (ESA), on November 1, 1999 (64 FR 58910) (USFWS 2002). The U.S. Fish and Wildlife Service (USFWS) is charged with developing federal recovery plans for listed bull trout. ***Distribution, abundance, habitat, and genetics are all considered important characteristics of population viability and recovery*** (my emphasis) (McElhaney et al. 2000).

Although bull trout are listed as Threatened by the Endangered Species Act, their distribution in Washington State is as yet adequately described. Those native char populations whose distributions are not accurately described are not likely to be adequately protected. This is especially true of resident native char subpopulations that live in stream headwaters upstream from barriers to anadromy, where they are reproductively isolated and where forest practices or impending changes in climate have extremely significant implications for their habitats and their survival. Funding for this project has been provided by the Western Native Trout Initiative and the Chehalis Tribe.

Overarching project goals:

- Collect tissue samples for genetic analysis of fish within purported resident bull trout distribution in the upper Wynoochee watershed, Grays Harbor County, WA.

- Analyze tissue samples and to identify species – performed by the WA Dept. of Fish and Wildlife in Olympia, WA.
- Prepare a report that includes the methodology, maps, photographs, and the results of the genetic analyses. The report will be shared with all interested agencies (USFWS, USFS, NOAA, WA Dept. of Fish and Wildlife, WA Dept. of Ecology, WA Dept. of Natural Resources), Tribes, and citizens. WFC project results are also reported online at www.wildfishconservancy.org .

Methods:

The Wynoochee River is a major tributary to the Lower Chehalis watershed (WRIA 22) in Grays Harbor County, WA. Using standardized protocols Wild Fish Conservancy (WFC) performed day (visual) and night (snorkel) surveys to locate representative fish in the upper Wynoochee watershed, where anecdotal observations of native char have been recorded (WDFW, pers. comm). Survey reaches were selected upstream from Wynoochee Falls, a full barrier to upstream fish passage, in part based on reach gradients of 1-2%. Survey protocols combined elements presented in Peterson *et al.* 2002 and Bonar *et al*. 1997. Surveys occurred in October 2012 and September 2013. Total stream channel length surveyed was approximately 3 miles; night snorkels were performed over a reach one-mile long.

During the night time snorkel survey, we brought representative fish to hand using dip nets. WFC collected photographs, underwater video, GPS landmarks, fish lengths, and genetic samples (caudal fin clip). All fish were released unharmed. The genetic samples were submitted to the WA Dept. of Fish and Wildlife Genetics laboratory in Olympia, WA for analysis.

Sampling Effort:

<u>October 20, 2012.</u>

Our three-person team performed an above-water reconnaissance of the upper Wynoochee River on October 20, 2012. Several hours after walking the reach in the daytime we conducted a night snorkel of the upper Wynoochee for the one half mile upstream from the Forest Service Road 2312 bridge at 47.49041N and 123.5292W (Figure 1). Surveyors included experienced staff from WFC and WDFW. Water temperature at the time of the survey was 6.1C. The team snorkeled the stream reach moving upstream between 8:00pm and 11:00pm. We brought three salmonids to hand: what appeared to be two Westslope cutthroat and one char. We collected a fin clip from the char, and stored it in an individually-labeled vial with 70% ETOH. Approximately ten sculpin were observed, but not brought to hand.

<u>September 7, 2013</u>

Our four-person team surveyed the upper Wynoochee River on September 7, 2013. Surveyors included experienced staff from WFC, WDFW, and USFS. Based on a daytime reconnaissance, a reach was identified for the nighttime snorkel survey (Figure 1) that started at the confluence of the mainstem Wynoochee and Chikamin Creek (Table 1).

Table 1. 2013 night snorkel survey extent.

	WGS84 datum	
	Latitude	Longitude
Upstream	47deg 30.186N	123deg 32.559W
Downstream	47deg 29.872N	123deg 32.155W

The team snorkeled the stream reach moving downstream between 8:30pm and 12:30am. WFC captured a total of twelve fish which appeared to be pure westslope cutthroat trout, and brook trout. One fish was a young-of-year, the others were sub-adults or adults. Approx. 15 sculpin were observed as well, but not brought to hand. Salmonids brought to hand were photographed, measured, and a tissue sample was collected from the caudal fin of seven representative fish – five char and two cutthroat. Each of the seven fin clips were preserved in 70% ETOH, each in their own individually-labeled vial.

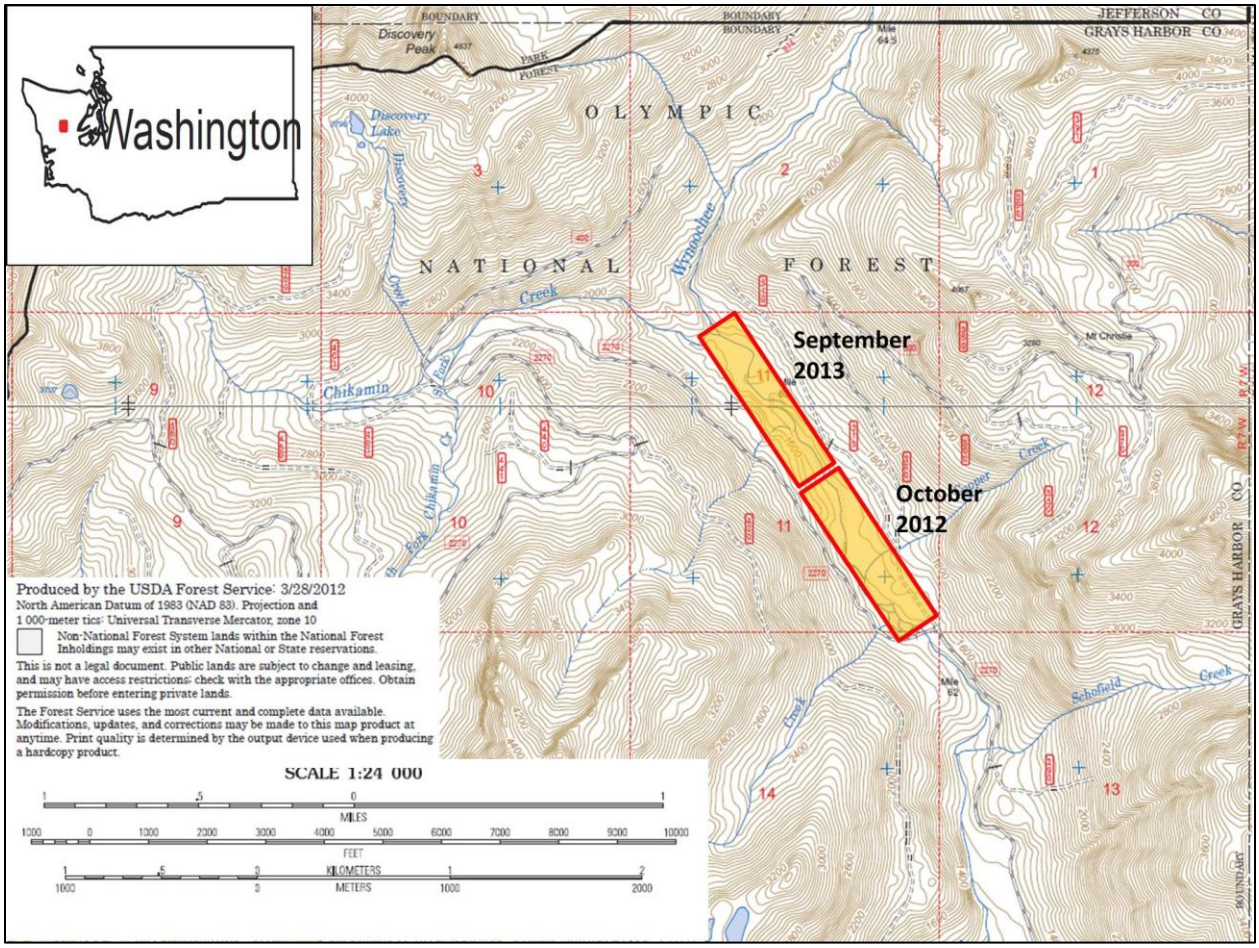

Figure 1. Upper Wynoochee night snorkel survey reaches.

Results and Discussion

We collected a total of eight fin clips from salmonids within the upper Wynoochee study area located upstream from Wynoochee Falls in Grays Harbor County, WA. Based on phenotypic characteristics observed in the field, six of the sampled fish appeared to be brook trout or possibly brook-bull trout hybrids; and two of the fish appeared to be westslope cutthroat trout. The WDFW Molecular Genetics Lab analysis of the collected fin clips confirmed we had sampled six brook trout and two westslope cutthroat trout (Appendix A and B).

In total we observed approximately sixty salmonids during the nighttime snorkel surveys; based on field identification their composition appeared to be evenly split between non-native brook trout and non-native westslope cutthroat trout. Additionally, we observed but did not capture approximately 25 native sculpin. We observed no native salmonids within the study reach during the surveys.

While we found no native char during the upper Wynoochee surveys, it is possible that an isolated headwater populations may exist further upstream from where we sampled. We do not consider this study conclusive on the question of whether native char exist in the upper Wynoochee watershed, and recommend surveys further upstream in future efforts. Furthermore, based on habitat and water temperatures observed in the adjacent upper Satsop headwaters, we recommend surveys be extended to include select portions of that watershed.

Figure 2. Wynoochee Falls, a complete barrier to upstream fish passage located downstream from the upper Wynoochee study reach. 47.480213N, -123.525847W.

Figure 3. Instream habitat typical of the Upper Wynoochee River, September 2013.

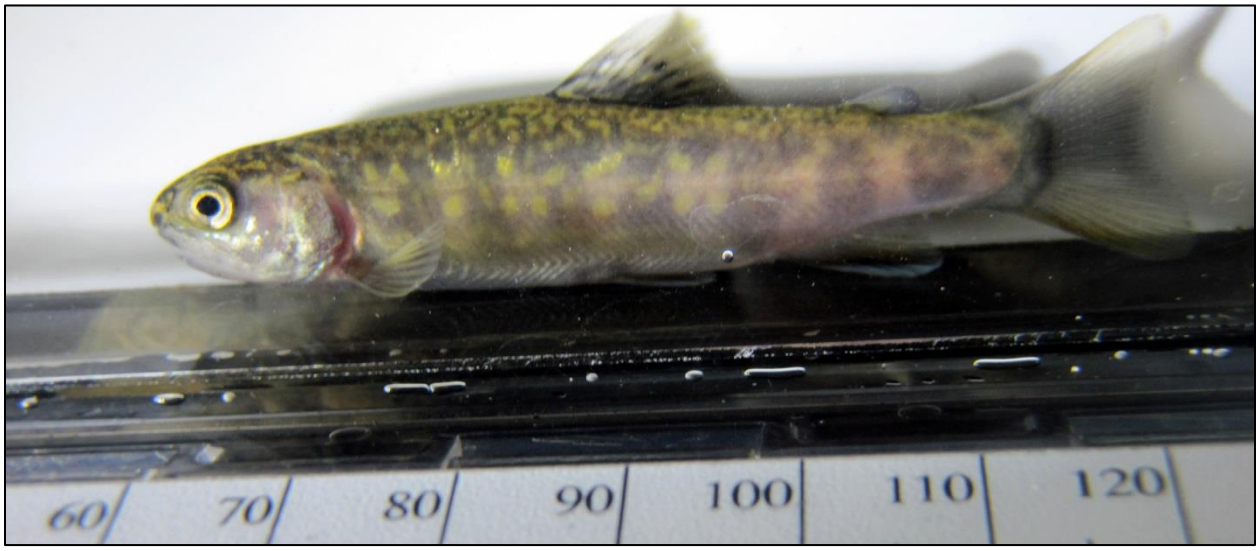

Figure 4. Juvenile brook trout brought to hand in the upper Wynoochee on October 20, 2012

Figure 5. ~200mm westslope cutthroat trout brought to hand in the Upper Wynoochee on September 7, 2013.

Figure 6. A 25mm young-of-year salmonid that was too small to fin clip.

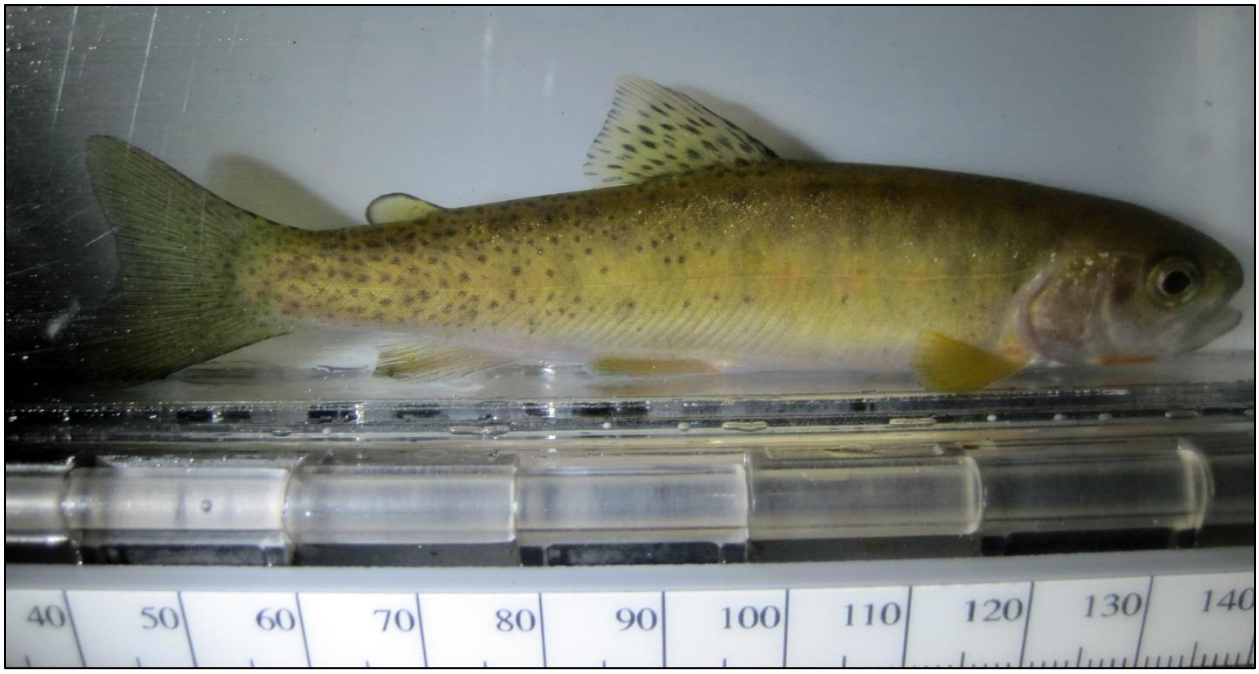

Figure 7. ~95mm westslope cutthroat trout brought to hand in the Upper Wynoochee on September 7, 2013.


Figure 8. ~160mm brook trout brought to hand in the Upper Wynoochee on September 7, 2013.

Appendix A.
WDFW Genetics Lab Report for One Char Sampled From the Upper Wynoochee in October 2012.

Genetic Analysis of an unknown char from the upper Wynoochee River, Washington

Maureen P. Small and Sonia Peterson
Molecular Genetics Lab, Conservation Unit, Science Division
Washington Department of Fish and Wildlife
600 Capitol Way N
Olympia, WA 98501
January 2013

In the work described here, we used a suite of 16 microsatellite DNA loci to evaluate the genetic identity of a char collected in the Wynoochee River. The objective of the work was:

- Identify sample as a bull trout, Dolly Varden, brook trout or hybrid (interspecific or interpopulation) using diagnostic genetic makers and comparison WDFW char genetic baseline.

Materials and Methods

Genotypic Data Collection

DNA was extracted from tissue sample using silica membrane kits (Macherey-Nagel). Fish was genotyped at 16 microsatellite DNA loci (Table 1) that are standardized among labs researching bull trout (Ardren et al. in prep). Microsatellite alleles were PCR-amplified using fluorescently labeled primers (see Table 2 for detailed PCR information). Primers that were fluorescently labeled with a vector tail (V) in our lab are identified in Table 1 by the label "V+a" after the primer (+a refers to a poly-a tail added to the reverse primer) and the concentration for the primer and the vector are given. The other primers were labeled at the factory when primers were constructed. PCRs were conducted in 384 well plates in 5 µl volumes employing 1 µl template with final concentrations of 1.5 mM $MgCl_2$, 200µM of each dNTP, and 1X Promega PCR buffer. After initial two minute denature at 94°, PCR temperature profiles followed a touchdown protocol (see Table 1) where the annealing temperature is lowered after an initial number of cycles. In the touchdown protocol the first four (profile 1) or 10 (profile 2) cycles have 94° denaturing for 30 seconds, 60° annealing for 30 seconds (decrease 1° per cycle) and extension at 72° for 60 seconds. These are followed by 36 (profile 1) or 30 (profile 2) cycles with the same parameters except for annealing temperature decreases to 50°, and then a final 10-minute extension at 72°. Samples were run on an ABI 3730 automated DNA Analyzer and

alleles were sized (to base pairs) and binned using an internal lane size standard (GS500Liz from Applied Biosystems) and GeneMapper software (Applied Biosystems).

Genetic Analyses

We used a Bayesian analysis implemented in STRUCTURE 2.2 (Pritchard et al. 2000) to estimate individual and possible hybrid ancestry. STRUCTURE sorts individuals (or portions of individuals if they are hybrids) into a number of hypothetical population clusters (K) in order to achieve Hardy-Weinberg equilibrium and linkage equilibrium in the clusters or populations. We compared the char to known bull trout, Dolly Varden and brook trout collections and observed which group it clustered with. Analysis was conducted in 5 independent runs that allowed admixture with 50,000 burn-ins and 100,000 iterations. The burn-in runs move the analysis away from the starting conditions to prevent them from influencing the analysis.

Results

Genotypic data collection

The unknown char amplified at a subset of bull trout loci and had brook trout-sized alleles at those loci.

Genetic analyses

STRUCTURE sorts individuals (or portions of individuals if they are of mixed ancestry) into genetically similar clusters. The unknown char clustered in the brook trout genetic cluster suggesting that it was a brook trout (Figure 1). There was no suggestion of hybridization.

Acknowledgements

The sample for this project was collected by Jamie Glasgow. Char baseline was funded by Puget Sound Energy, University of Washington, Seattle City Light, and Washington State General Funds.

References

Ardren, W. R., P. W. DeHaan, P. Spruell, C. Bettles, R. Taylor, C. Cegelski, M. Campbell, P. Tamkee. (in preparation). Bull trout locus and allele standardization.

Pritchard, J. K., M. Stephens, and P. Donnelly. (2000). Inference of population structure using multilocus genotype data. Genetics 155:945-959.

Figure 1. Individual ancestry values from STRUCTURE for K = 3 including Baker basin bull trout, brook trout collected in lower Baker basin, and Dolly Varden collected in Diablo Lake in the upper Skagit basin. Each cluster (genetic group or population) has an associated color (e.g. blue for Dolly Varden); single color for an individual suggests pure ancestry and multiple colors suggest mixed ancestry (ancestry in more than one cluster). The unknown char is at the top of the plot and has 100% ancestry in the brook trout cluster.

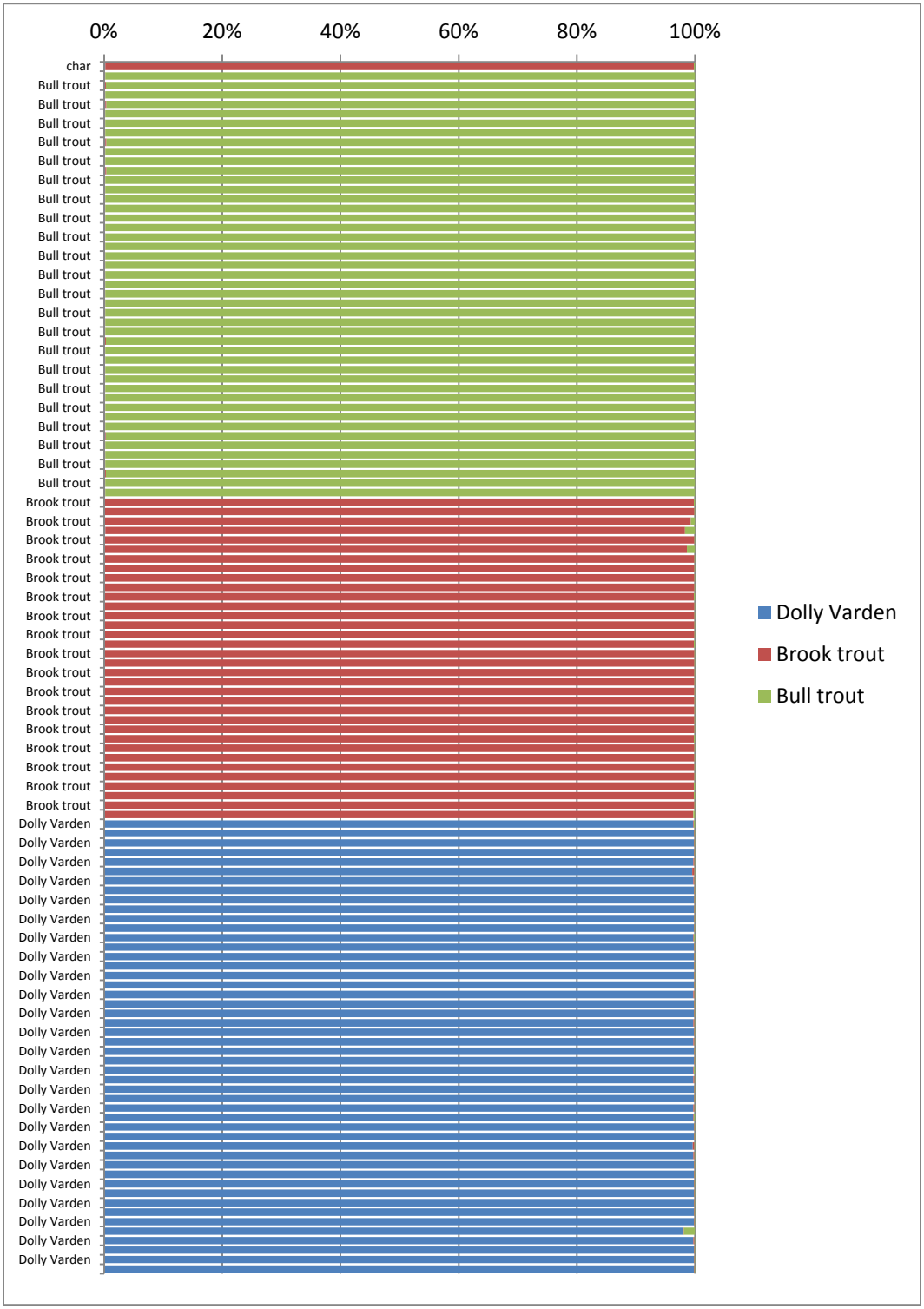

Table 1. Information for multiplexes and loci including annealing temperature (°C) and primer and vector concentration. Annealing profiles are detailed below table. References for primer sequences are under Citation. The +a indicated a poly-a tail on the primer.

Multiplex	Locus	Anneal profile	conc [uM]	Citation
Sco-E	Omm-1128 +a	1	0.14	Rexroad et al. (2001)
	Sco-105 +a	1	0.08	WDFW unpublished data
Sco-1.1	Sco-218 V1+a	2	0.16	DeHaan and Ardren (2005)
	V1		0.08	
	Sco-202 V2+a	2	0.13	DeHaan and Ardren (2005)
	V2		0.06	
	Sco-200 V4+a	2	0.21	DeHaan and Ardren (2005)
	V4		0.1	
Sco-1.2	Sco-220 V3+a	2	0.12	DeHaan and Ardren (2005)
	V3		0.06	
Sco-J	Sco-216 V2+a	2	0.16	DeHaan and Ardren (2005)
	V2		0.08	
	Sco-215 V4+a	2	0.11	DeHaan and Ardren (2005)
	V4		0.05	
Sco-K	Sco-109 +a	2	0.26	WDFW unpublished data
	Sfo-18 V3 +a	2	0.14	Angiers and Bernachez (1996)
	V3		0.07	
	Smm-22 V4 +a	2	0.17	Crane et al. (2004)
	V4		0.08	
Sco-L	Sco-106 +a	1	0.14	WDFW unpublished data
	Sco-102 +a	1	0.07	WDFW unpublished data
	Omm-1130 +a	1	0.15	Rexroad et al. (2001)
Sco-M	Sco-212 V2 +a	1	0.16	DeHaan and Ardren (2005)
	V2		0.08	
	Sco-107 +a	1	0.13	WDFW unpublished data

Anneal profile 1: 4 cycles 94º for 30 sec, 60º anneal for 30 sec (decrease 1° per cycle), 72º for 60 sec then 36 cycles with 50º anneal (no decrease)

Anneal profile 2: 10 cycles 94º for 30 sec, 60º anneal for 30 sec (decrease 1° per cycle), 72º for 60 sec then 30 cycles with 50º anneal (no decrease)

Appendix B.
WDFW Genetics Lab Report for Seven Salmonids Sampled From the Upper Wynoochee in September 2013.

Genetic Analysis of unknown trout and char from the upper Wynoochee River, Washington

Maureen P. Small and Vanessa Smilansky
Molecular Genetics Lab, Conservation Unit, Science Division
Washington Department of Fish and Wildlife
600 Capitol Way N
Olympia, WA 98501
February 2014

Introduction

Seven trout were collected in the Wynoochee River in summer 2013. Phenotypic identity was difficult and we used genetic analysis to identify fish to species and determine whether fish were hybrids. Two fish appeared to be trout, possibly cutthroat, and five appeared to be char, so all fish were genotyped with seven microsatellite loci used to identify cutthroat trout and to distinguish coastal cutthroat and rainbow trout, and 16 loci used to identify char. Then fish were compared to baseline collections consisting of coastal and Westslope cutthroat trout and coastal rainbow trout, and another baseline consisting of collections of bull trout, brook trout, Arctic char and Dolly Varden.

The objective of the work was:

- Identify samples as coastal or Westslope cutthroat trout, coastal rainbow trout, bull trout, Dolly Varden, Arctic char, brook trout or hybrids (interspecific or interpopulation) using diagnostic genetic markers and comparison WDFW trout and char genetic baselines.

Materials and Methods

Genotypic Data Collection

DNA was extracted from tissue sample using silica membrane kits (Macherey-Nagel). Fish were genotyped at seven microsatellite loci (Table 1) that were used to identify and distinguish between cutthroat and rainbow trout in WDFW studies (Marshall et al. 2006, Thompson et al. 2011). Fish were also genotyped at 16 microsatellite DNA loci (Table 2) that are standardized among labs researching bull trout (Ardren et al. in prep). Microsatellite alleles were PCR-amplified using fluorescently labeled primers (see Table 2 for detailed PCR information). Primers that were fluorescently labeled with a vector tail (V) in our lab are identified in Table 1 by the label "V+a" after the primer (+a refers to a poly-a tail added to the reverse primer) and the concentration for the primer and the vector are given. The other primers were labeled at the factory when primers were constructed. PCRs were conducted in 384 well plates in 5 µl volumes employing 1 µl template with final concentrations of 1.5 mM $MgCl_2$, 200µM of each dNTP, and 1X Promega PCR buffer. After initial two minute denature at 94°, PCR temperature profiles followed a touchdown protocol (see Table 2) where the annealing temperature is lowered after an initial number of cycles. In the touchdown protocol the first four (profile 1) or 10 (profile 2) cycles have 94° denaturing for 30 seconds, 60° annealing for 30 seconds (decrease 1° per cycle) and extension at 72° for 60 seconds. These are followed by 36 (profile 1) or 30 (profile 2) cycles with the same parameters except for annealing temperature decreases to 50°, and then a final 10-minute extension at 72°. Samples were run on an ABI 3730 automated DNA Analyzer and alleles were sized (to base pairs) and binned

using an internal lane size standard (GS500Liz from Applied Biosystems) and GeneMapper software (Applied Biosystems).

Genetic Analyses
We used a Bayesian analysis implemented in STRUCTURE 2.2 (Pritchard et al. 2000) to estimate individual and possible hybrid ancestry. STRUCTURE sorts individuals (or portions of individuals if they are hybrids) into a number of hypothetical population clusters (K) in order to achieve Hardy-Weinberg equilibrium and linkage equilibrium in the clusters or populations. We compared the trout to a baseline consisting of Westslope cutthroat trout from Twin Lakes Hatchery, coastal cutthroat trout from North and Central Puget Sound, and coastal rainbow trout from Puget Sound. We compared the char to bull trout from the coast, Puget Sound and Columbia River, Dolly Varden from the coast and Puget Sound, hatchery brook trout, and two hatchery Arctic char collections, and in both analyses observed which group the trout clustered with. Analyses were conducted in 5 independent runs that allowed admixture with 50,000 burn-ins and 100,000 iterations. The burn-in runs move the analysis away from the starting conditions to prevent them from influencing the analysis.

Results
Genotypic data collection
The two unknown trout amplified at the cutthroat trout loci, failed at 12/16 char loci, and had odd-sized alleles (alleles outside the size range for char) at 4/16 char loci.
The five unknown char amplified at 13/16 bull trout loci and had brook trout-sized alleles at those loci. They also amplified at 3/7 cutthroat loci and were fixed for odd-sized alleles (alleles outside the size range for cutthroat and rainbow trout) at those loci.

Genetic analyses
STRUCTURE sorts individuals (or portions of individuals if they are of mixed ancestry) into genetically similar clusters.

- The two unknown trout (13MP3, 13MP5) clustered with the Westslope cutthroat trout from Twin Lakes Hatchery, suggesting they were hatchery Westslope cutthroat trout (Figure 1). There was no suggestion of hybridization
- The five unknown char (13MP1, 13MP2, 13MP4, 13MP6, 13MP7) clustered in the brook trout genetic cluster suggesting they were brook trout (Figure 2). There was no suggestion of hybridization.

Acknowledgements
The samples for this project were collected by Jamie Glasgow. Trout baseline was funded by Puget Sound Energy and Washington State General Funds. Char baseline was funded by Puget Sound Energy, University of Washington, Seattle City Light, and Washington State General Funds.

References
Ardren, W. R., P. W. DeHaan, P. Spruell, C. Bettles, R. Taylor, C. Cegelski, M. Campbell, P. Tamkee. (in preparation). Bull trout locus and allele standardization.

Marshall, A. M., M. P. Small, and S. Foley. 2006. Genetic relationships among anadromous and non-anadromous *Oncorhynchus mykiss* in Cedar River and Lake Washington - implications for steelhead recovery planning. Final Report to Cedar River Anadromous Fish Committee and Seattle Public Utilities.

Pritchard, J. K., M. Stephens, and P. Donnelly. (2000). Inference of population structure using multilocus genotype data. Genetics 155:945-959.

Thompson, J. N, M. P. Small, and C. Dean. 2011. Genetic composition of Pacific trout species in relation to landscape features in the upper Snoqualmie River watershed, WA

Figure 1. Individual ancestry values from STRUCTURE for K = 5 including Westslope cutthroat trout from Twin Lakes Hatchery, Puget Sound rainbow trout (Omy), two Puget Sound coastal cutthroat trout populations from the Cedar and Snoqualmie Rivers, and one coastal cutthroat trout population from Lake Whatcom in North Puget Sound that had been used as a hatchery broodstock for hatchery coastal cutthroat trout. Each cluster (genetic group or population) has an associated color (e.g. dark blue for Westslope); single color for an individual suggests pure ancestry and multiple colors suggest mixed ancestry (ancestry in more than one cluster). The unknown trout are at the top of the plot and have 100% ancestry in the Westslope cluster.

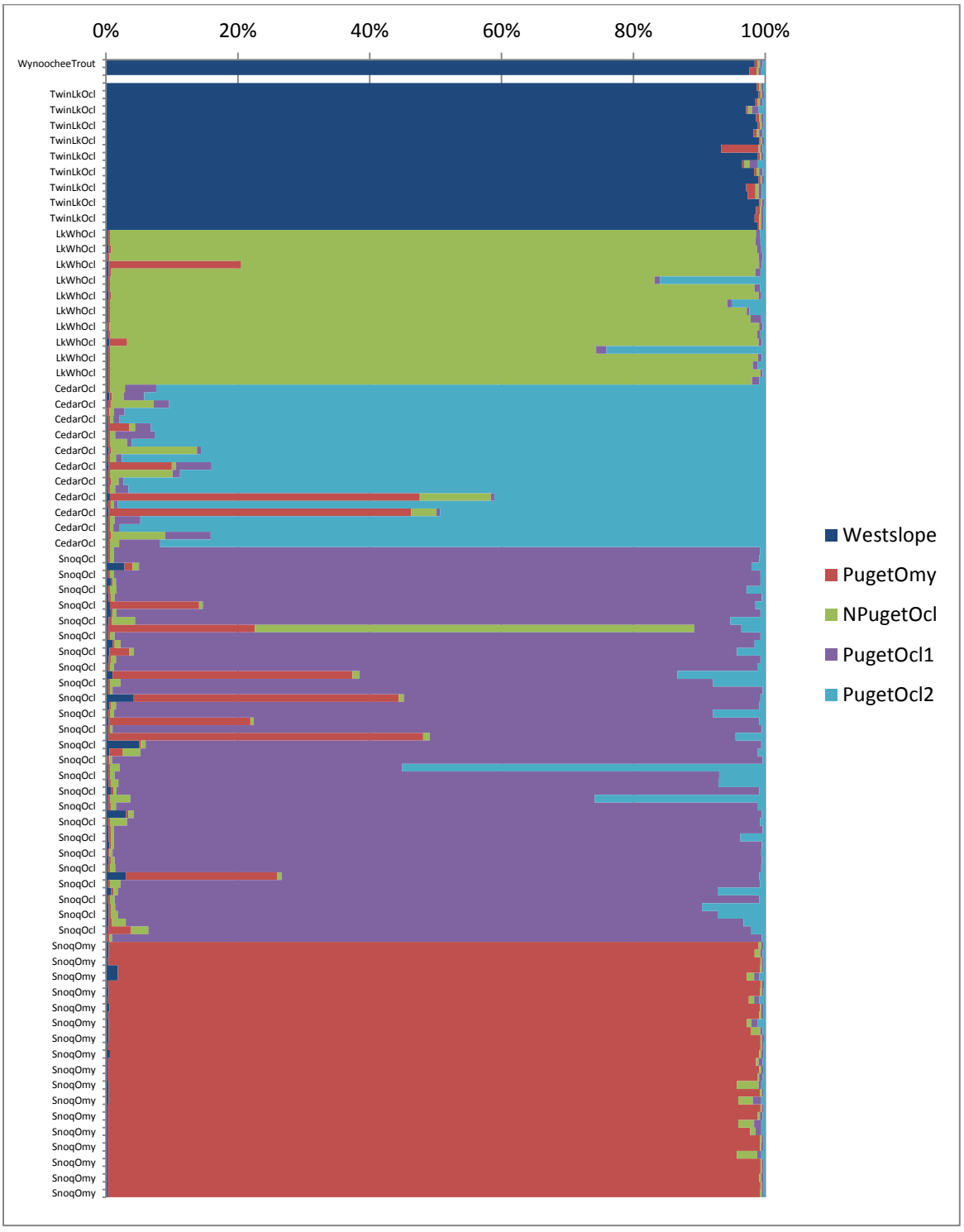

Figure 2. Individual ancestry values from STRUCTURE for K = 5 including brook Trout, bull trout from Puget Sound, Lower Columbia (LoColum), and the coast (Quinault "Q", and Hoh rivers), Dolly Varden collected in Diablo Lake in Puget Sound and the coast (Sol Duc River), and two hatchery Arctic char collections. Each cluster (genetic group or population) has an associated color (e.g. blue for Dolly Varden); single color for an individual suggests pure ancestry and multiple colors suggest mixed ancestry (ancestry in more than one cluster). The five unknown char are at the top of the plot and have 100% ancestry in the brook trout cluster.

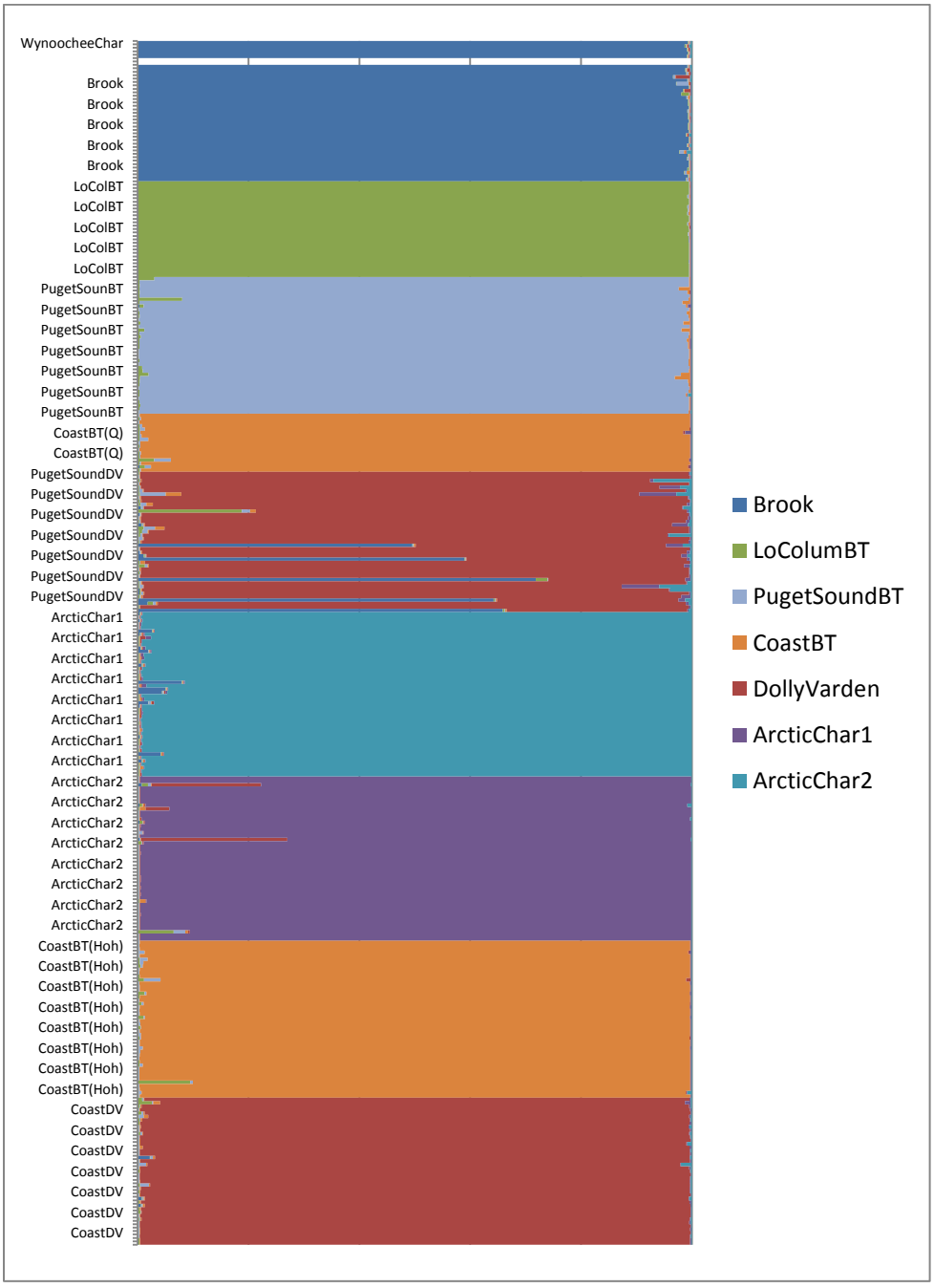

Table 1. Information for multiplexes and loci. PCR's were conducted on a MJResearch PTC-200 thermocycler in 10 μl volumes employing 1 μl template with final concentrations of 1.5 mM $MgCl_2$, 0.05 units of *Taq* polymerase and 1X Promega PCR buffer. Number of PCR cycles is under "cycles".

Multiplex	Anneal T	cycles	Locus
Omy-C2	55	28	One-108
			Ots-103
Omy-D2	49	25	Ots-1
			Omy-77
			Ots-3M
			Ogo-3
Ocl-F2	50	35	Omm-1138

Table 2. Information for multiplexes and loci including annealing temperature (°C) and primer and vector concentration. Annealing profiles are detailed below table. References for primer sequences are under Citation. The +a indicated a poly-a tail on the primer.

Multiplex	Locus	Anneal profile	conc [uM]	Citation
Sco-E	Omm-1128 +a	1	0.14	Rexroad et al. (2001)
	Sco-105 +a	1	0.08	WDFW unpublished data
Sco-1.1	Sco-218 V1+a	2	0.16	DeHaan and Ardren (2005)
	V1		0.08	
	Sco-202 V2+a	2	0.13	DeHaan and Ardren (2005)
	V2		0.06	
	Sco-200 V4+a	2	0.21	DeHaan and Ardren (2005)
	V4		0.1	
Sco-1.2	Sco-220 V3+a	2	0.12	DeHaan and Ardren (2005)
	V3		0.06	
Sco-J	Sco-216 V2+a	2	0.16	DeHaan and Ardren (2005)
	V2		0.08	
	Sco-215 V4+a	2	0.11	DeHaan and Ardren (2005)
	V4		0.05	
Sco-K	Sco-109 +a	2	0.26	WDFW unpublished data
	Sfo-18 V3 +a	2	0.14	Angiers and Bernachez (1996)
	V3		0.07	
	Smm-22 V4 +a	2	0.17	Crane et al. (2004)
	V4		0.08	
Sco-L	Sco-106 +a	1	0.14	WDFW unpublished data
	Sco-102 +a	1	0.07	WDFW unpublished data
	Omm-1130 +a	1	0.15	Rexroad et al. (2001)
Sco-M	Sco-212 V2 +a	1	0.16	DeHaan and Ardren (2005)
	V2		0.08	
	Sco-107 +a	1	0.13	WDFW unpublished data

Anneal profile 1: 4 cycles 94º for 30 sec, 60º anneal for 30 sec (decrease 1° per cycle), 72º for 60 sec then 36 cycles with 50º anneal (no decrease)

Anneal profile 2: 10 cycles 94º for 30 sec, 60º anneal for 30 sec (decrease 1° per cycle), 72º for 60 sec then 30 cycles with 50º anneal (no decrease)