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Rapid PCR-based species tests for threatened sympatric salmonids

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Molecular diagnostic tests provide valuable information for conservation management, but those involving multiple steps are unduly laborious for the analysis of large numbers of samples. Fortunately, PCRs can be designed to detect diagnostic polymorphisms directly.

Three salmon species, coho (*Oncorhynchus kisutch*), chinook (*O. tshawytscha*) and steelhead (*O. mykiss*), are sympatric over a wide range of the Pacific northwest, and encompass ESUs listed as threatened or endangered (NMFS 2001). PCR-based markers used for the characterization of populations to provide information to managers also amplify in sympatric species, and occasionally, unusual genotypes complicate analysis, indicating the need for efficient molecular species verification. Also, morphologically unidentifiable samples are otherwise unusable. Previously developed techniques to classify Pacific salmon (Devlin 1993; McKay et al. 1997; Withler et al. 1997) require two or more post-amplification restriction enzyme digests.

Here we present two accurate and rapid species tests. *I-Ots-2* is a microsatellite isolocus: a duplicated locus presumably surviving from the salmonid tetraploid event of 25–100My ago (Allendorf and Thorgaard 1984). It was co-amplified by primers designed when incorporating *Ots-2* into a multiplexed set (Greig and Banks 1999), and its species-distinct allele profiles were immediately apparent. Alleles were identified and sequenced in several chinook populations and four other *Oncorhynchus* species. All chinook populations analysed were monomorphic, with identical sequences. A single ‘G’ not seen in *Ots-2* interrupts the microsatellite repeat, reducing the direct repeat number to four. Steelhead have a larger common allele with seven uninterrupted repeats, and two others with six and nine uninterrupted repeats respectively. In coho, the locus is larger and highly variable, with 15–20 uninterrupted repeats. Perhaps unusually for a microsatellite, the band patterns for each species are distinctive (Figure 1A).

The second test was designed to facilitate rapid typing, and detects growth hormone type-2 sequence differences among species (McKay et al. 1997). A G/C polymorphism between chinook and coho/steelhead at position 327 of intron D (McKay et al. 1996) was chosen for primer development, using criteria including base mismatch, Tm, and GC content based on ‘ARMS’ (Newton et al. 1989) and ‘ASPCR’ (Wu et al. 1989). The PCR product also encompasses an 8bp indel between steelhead and chinook/coho. Primers were extended to achieve matching G/C content and Tm, with the chinook-specific primer 4bp longer than that for coho/steelhead. These two reverse primers were 5′-labeled with different fluorophores. This resulted in a single-tube PCR reaction whose product was either a 124bp-steelhead allele or 116bp-coho allele seen at 505nm, or a 120bp-chinook allele seen at 585nm (Figure 1). Reactions were optimised for annealing temperature, and concentrations of magnesium, dNTPs and primers. The 3-primer reaction was robust; slight cross-amplification seen in 2-primer PCRs was eliminated by reducing primer and dNTP concentrations.

Primers initially developed for multiplexing the microsatellite *Ots-2* were used for *I-Ots-2* (*I-Ots-2 F*: 5′Hex-GAGCCGCAACAATGTAAATG 3′, *I-Ots-2 R*: 5′ GCGAGCTGAATGTAAAGATGAC 3′). 10 μl PCR mixes contained $1 \times$ Buffer (Promega), 1.625 mM $MgCl_2$, 0.125 mM dNTPs, 0.5 μM primers, 0.025U Taq polymerase (Promega), and 2 μl DNA.

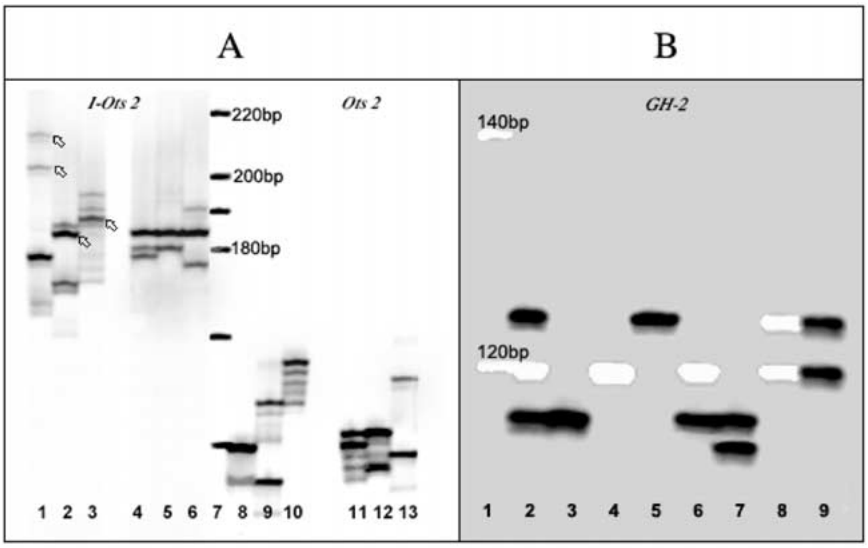

Figure 1. Showing typical results for: **A**: *I-Ots-2* test: Isolocus bands are indicated by an arrow (not present in *Ots 2* amplified alone). (1) Coho, showing distinctive isolocus bands above 200bp, (2) Chinook, with distinctive 183bp band, (3) Steelhead, (4–6) Unknown samples types as chinook, (7) Size ladder, (8–13) As 1–6, *Ots 2* bands alone for comparison. **B**: *GH-2* test: Bands visualised at 505nm are black, those visualised at 585nm are white. (1) Size ladder, (2) Mixed coho, chinook and steelhead, (3) Coho, (4) Chinook, (5) Steelhead, (6) Chinook/coho hybrid, (7) Coho showing polymorphism, (8) Chinook showing polymorphism, (9) Steelhead showing polymorphism.

Cycling was in 96-well plates in an MJ thermocyler (MJ Research, Inc.) at 94 °C-3 min, 57 °C-30 s, 72 °C-30 s, followed by 35 cycles of 94 °C-30 s, 57 °C-20 s, 72 °C-30 s, then 72 °C-2 min. GH2 primer sequences have $5'$ extensions underlined: *GH2onchF*: $5'$-AATTCCAGCATGCTCTACTACAGG$3'$, *GH2chinR*: $5'$Cy3 – GCTCCTATATATCTGTGTGTAGCATAAGATC$3'$, *GH2cohoR*: $5'$ FAM-GCGTATATCTGTGTGTAGCATAAGATG$3'$. 10 μl PCR mixes contained $1 \times$ Buffer (Promega), 1.25 mM $MgCl_2$, 0.075 mM dNTPs, 0.2μM primers Onch-F, Chin-R, 0.16μM primer Coho-R, 0.025U Taq polymerase (Promega), and 2 μl DNA. Cycling was as above with an annealing temperature of 60 °C.

A large baseline of adult fish ($n > 100$, at least 5 populations) was typed for each species, together with 40 of unknown species (sample sizes and genotypes provided in Table 1); also hybrid coho/chinook DNA provided by R. H. Devlin (personal communication). DNA was extracted using 5% Chelex® (BioRad) or the Puregene® isolation kit (Gentra Systems) (Banks et al. 1999).

Ninety-six PCRs were run on each 8% denaturing polyacrylamide gel at 50W for 1 h 45 min (*I-Ots-2*) or 1 h 20 min (*GH-2*) and products visualised using an Hitachi FMBIO II laser scanner at 585nm (*I-Ots-2*) or 585nm and 505nm (*GH-2*). Genotypes for both tests are shown (Figure 1). *I-Ots-2* genotypes were determined by size comparison with *Ots-2* alleles amplified alone (Figure 1A).

The expected species-specific alleles for *I-Ots-2* were identified in known adult chinook, coho and steelhead, sampled in a range of populations (Table 1). For *GH-2*, the expected alleles were the most commonly observed in all species; but rarer alleles differing in size were also seen. This is consistent with a trend for intron deletions at the interspecies level in this gene (Devlin 1993). Additional alleles were seen in chinook at 124bp, coho at 114bp and in steelhead at 120bp. These were usually observed in the heterozygous state with the common allele. This resulted in size overlaps in a small number of samples between chinook and steelhead. However the G/C sequence difference is fixed between species, so alleles are still distinguishable by colour, resulting in an easy-to-score band pattern, and reliable species identification.

Our results show both tests are diagnostic for discrimination among *O. kisutch*, *O. tshawytscha*, and *O. mykiss* (Figure 1). The tests gave equivalent results in agreement with morphological identification of known species and unambiguously classified all unknown fish.

The tests continue to prove their utility in our laboratory. For example, eleven fish from the Russian

Table 1.

I-Ots-2 genotypes: number of fish observed										
Chinook										
Sample:	CA-1	CA-3	CA-5	OR-1	AK-2					Total
Genotype*	$n = 24$	$n = 45$	$n = 24$	$n = 24$	$n = 24$					$n = 141$
183/183	24	45	24	24	24					100%
200+/200+										
187+/187+										
Coho										
Sample:	CA-3	CA-7	CA-14	AK-3	BC-2	BC-3	WA-1	WA-2	RU-1	Total
Genotype*	$n = 41$	$n = 48$	$n = 65$	$n = 12$	$n = 12$	$n = 11$	$n = 12$	$n = 12$	$n = 12$	$n = 214$
183/183										
200+/200+	41	48	65	12	12	11	12	12	12	100%
187+/187+										
Steelhead										
Sample:	CA-3	CA-4	CA-5	AK-4	AK-5	BC-4	BC-5			Total
Genotype*	$n = 39$	$n = 67$	$n = 27$	$n = 15$	$n = 15$	$n = 15$	$n = 15$			$n = 193$
183/183										
200+/200+										
187+/187+	39	67	27	15	15	15	15			100%
Hybrid (Coho/Chinook)										
Genotype*	$n = 1$									
183/215	1									100%
Unknown										
Sample:	CA-6	CA-9	CA-10	CA-11	CA-12					Total
Genotype*	$n = 7$	$n = 1$	$n = 19$	$n = 1$	$n = 11$					$n = 40$
Typed as Chinook										
183/183		1	2	1	11					
200+/200+										38%
Typed as Coho										
200–250	2		17							50%
Typed as Steelhead										
187+/187+	5									13%

GH-2 genotypes: number of fish observed											
Chinook											
Sample:	CA-1	CA-2	CA-3	AK-1	BC-1						Total
Genotype*	$n = 42$	$n = 45$	$n = 36$	$n = 19$	$n = 86$						$n = 228$
120C/120C	33	29	35	19	78						85.1%
124C/120C	8	16	1		8						14.5%
124C/124C	1										0.4%
Coho											
Sample:	CA-3	CA-7	CA-8	CA-9	WA-1	WA-2	AK-3	BC-2	BC-3	RU-1	Total
Genotype*	$n = 48$	$n = 57$	$n = 8$	$n = 22$	$n = 12$	$n = 12$	$n = 12$	$n = 12$	$n = 12$	$n = 12$	$n = 207$
116F/116F	37	41	8	15	9	5	3	6	3	3	62.8%
116F/114F	11	14		7	3	6	6	6	9	7	33.3%
114F/114F		2				1	3			2	3.9%
Steelhead											
Sample:	CA-3	CA-4	CA-5	CA-6	AK-4	AK-5	BC-4	BC-5			Total
Genotype*	$n = 29$	$n = 44$	$n = 30$	$n = 1$	$n = 15$	$n = 15$	$n = 15$	$n = 15$			$n = 164$
124F/124F	23	15	21	1	4	12	5	4			51.8%
124F/120F	6	25	7		6	3	10	10			40.9%
120F/120F		4	2		5			1			7.3%
Hybrid (Coho/Chinook)											
Genotype*	$n = 1$										
120C/116F	1										100%
Unknown											
Sample:	CA-6	CA-9	CA-10	CA-11	CA-12	CA-13					Total
Genotype*	$n = 7$	$n = 1$	$n = 19$	$n = 1$	$n = 11$	$n = 1$					$n = 40$
Typed as Chinook											
120C/120C		1			11						30.0%
124C/120C			2								5.0%
120C/116C				1							2.5%
Typed as Coho											
116F/116F	2		17								47.5%
114F/114F						1					2.5%
Typed as Steelhead											
124F/124F	5										12.5%

*Base pairs, 200+ = alleles of size 200–250bp, 187+ = alleles of size 187, 189, or 193bp, F = Fam, C = Cy3. Sample locations: CA-1, California Central Valley:Winter, Spring, Fall, and Late Fall runs; CA-2, Nimbus Hatchery, American River, California; CA-3, Warm Springs Hatchery, Russian River, California; CA-4, Rowdy Creek Hatchery, Smith River, N. California; CA-5, Klamath River, N. California; CA-6, Wohler Pond, Russian River, California; CA-7, Noyo Egg Taking Station, Noyo River, California; CA-8, Lagunitas Creek, Marin, California; CA-9, Olema Creek, Pt.Reyes Natl Seashore, Marin, California; CA-10, Lower Trinity River, N. California; CA-11, Mirabel Pond, Russian River, California; CA-12, Russian River Estuary, California; CA-13, E. Fork Mill Creek, Smith River, N.California; CA-14, Greenvalley Creek, Russian River, California; OR-1, Alsea River, Oregon; WA-1, Samish Bay river, Puget sound North, Washington; WA-2: Hood Canal, Puget sound South: Washington; BC-1, Harrison River, Fraser watershed, British Columbia, Canada; BC-2, Maur River, British Columbia; BC-3, Skeena-Lachman Fence, British Columbia; BC-4, Coquihalla river, Lower Fraser system, British Columbia; BC-5, Kwinageese river, Nass system, British Columbia; AK-1, S. Alaska (Tahini, Chikamin, Togiak, Unuk, King Salmon Hatchery); AK-2, Unuk River, Alaska; AK-3, Hidden Falls, S.E. Alaska; AK-4, Sturgeon River, Kodiak Island Alaska; AK-5, Kodiak river, Kodiak Island Alaska; RU-1, Kamchatka Saichi, Russia.

River (sample CA-12) were originally thought to be coho smolts, but had unusual microsatellite genotypes. We were able to reveal that these samples represented the first record in over 50 years of wild-spawned chinook in the Russian River in California. We have also been able to easily identify pinniped prey from 'feeding event' debris, important for stock impact assessment.

Nuclear DNA markers should also detect chinook/coho hybridization which may occur in the wild, or inadvertently in hatcheries (Chevassus 1979; Bartley et al. 1990). We typed one experimentally created chinook/coho hybrid, and in both tests observed one allele specific to each species (Figure 1).

In conclusion, we have developed two rapid tests for reliable species diagnosis. Both have been extensively characterised against baseline samples from across the Pacific northwest, and show consistent among-species differences. They are suitable for high throughput and can be used with a minimal, non-invasively obtained tissue sample. Together they provide the confirmation of results required in special cases, such as identifying fish from watersheds where a species is thought to be absent, or confirming the identity of suspected hybrids. Their accuracy and ease of use makes them a useful addition to the molecular tools used in salmon conservation biology.

Genbank accession numbers
Ots-2 [AF107030].
GH-2 [U2815] (chinook), [U28359] (coho), [J0397] (steelhead).

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