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Do Dead Fish Tell Tales? DNA Degradation in Chinook Salmon (*Oncorhynchus tshawytscha*) Carcasses

Abstract

Carcasses are potentially a valuable source of DNA for genetic studies of Pacific salmon (*Oncorhynchus sp.*). They can be collected at spawning grounds across a wide geographic area and include populations in streams in which it is logistically difficult or too intrusive to sample spawning fish. However, the quality and quantity of DNA from salmon carcasses is highly variable. Using a standardized set of microsatellite loci, we investigated the relationship of amplification success and genotyping errors to time since death and locus size in Chinook salmon (*O. tshawytscha*) carcasses. Amplification success declined rapidly from death, smaller loci had greater amplification success than larger loci, and genotyping errors were present in 5% of scored samples. Salmon carcasses can be a valuable source of genetic information; however, the level of effort needed to produce accurate and reliable data with microsatellite loci is considerable. Ideally, field collections should be frequent and focus on fresh carcasses. Data replication should also be incorporated into analyses to reduce amplification failures and genotyping errors. New techniques and markers should improve future cost-effectiveness when analyzing degraded tissue samples. However, we recommend that an evaluation of cost and time be performed at the onset of any population genetic study using salmon carcass tissues, regardless of the technique used.

Introduction

Non-invasive genetic sampling (NGS) is desirable for the study of threatened and endangered species because it is a non-intrusive and logistically feasible way to collect data from free-ranging animals. NGS techniques have proliferated such that genotypes now can be determined from low quantity DNA sources such as hair, feces, urine, feathers, eggs shells, sloughed skin, and saliva (see review in Waits and Paetkau 2005). These techniques have provided valuable and, in some cases, otherwise unobtainable genetic data for studies addressing population estimation, population genetic structure, and genetic diversity in rare, elusive, or remote mammals (Taberlet et al. 1997, Piggot and Taylor 2003, Hedmark et al. 2004, Swanson et al. 2006). The use of NGS techniques for aquatic animals, namely fish species, has similar appeal. However, Pacific salmon (*Oncorhynchus sp.*) spend most of their adult life in the ocean and die after spawning, so opportunities to sample at the population level are limited. Further, many

threatened and endangered salmon populations spawn in remote locations where sampling is logistically difficult. Many salmon genetic studies rely on permanent or semi-permanent structures such as weirs, emigrant traps, or fishing methods to collect non-destructive fin clips from fish. Carcasses of spawned-out adults are potentially a valuable non-invasive source of DNA for salmon population genetic studies. Obtaining information from adult salmon without disturbing spawning and sampling remote populations without expensive infrastructure are two attractive features of NGS to salmon conservation biologists.

Many NGS studies have encountered problems such as high error rates, low success rates, and contamination associated with the concentration and quality of the template DNA (Taberlet et al. 1996, Goossens et al. 1998, Taberlet et al. 1999, Creel et al. 2003, Broquet and Petit 2004, Broquet et al. 2006). Genotype errors can lead to biased estimates of genetic diversity and population size (Taberlet and Luikart 1999, Waits and Leberg 2000). Such errors can be controlled through data replication (Taberlet et al. 1997, Paetkau 2003, Waits and Paetkau 2005). However, the

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costs associated with data replication can be 5 to 10-fold higher than with fresh tissue (Taberlet et al. 1999). Costs and the ability to obtain accurate genetic information are important when determining the appropriateness of a noninvasive source of genetic material. Given the technical difficulties associated with amplification of NGS, Taberlet et al. (1999) advised performing studies to estimate amplification success and error rates. This information can be used to determine costs and feasibility for NGS techniques.

Previous investigators found variable DNA quality in samples from salmon carcasses that affected genotyping success. In a large study examining the population structure of Chinook salmon (*O. tshawytscha*) in the Middle Fork Salmon River, Idaho, Neville et al. (2006a) indicated that the DNA quality from carcasses was low due to degradation in the field and needed to replicate each sample three times for quality control. Avelino (1997) also had difficulty amplifying DNA from Chinook salmon carcass tissue samples, which limited the number of samples available for analysis. In her study, carcass tissue samples provided half as much genetic data as fresh tissue. Collection of tissues from salmon carcasses for genetic analyses is a routine part of salmon monitoring studies (Crawford et al. 2007). Currently, the Idaho Department of Fish and Game (IDFG) maintains a large archive of salmon carcass tissues. The technical difficulties described by these studies motivated us to examine factors influencing the probability of obtaining accurate genotypes from carcass samples and to evaluate the utility of this large archive. We also sought to determine if future field collection or laboratory methods could be adjusted to provide samples with higher-quality DNA.

We present a study using Chinook salmon carcasses from two hatcheries in Idaho. In this study, we assessed the effect of DNA degradation in salmon carcasses on our ability to obtain genetic information. Our primary question was: How fast does amplification success decline after death? We assumed that salmon adults that just spawned and died would have fresh tissue and determined the relationship of amplification success to location, microsatellite loci used, and carcass age (e.g., time since death). The goal of this study was to provide guidance for future collection and laboratory processing of samples. Based on the results reported here, we propose guidelines and

discuss limitations for using carcass samples in salmon population genetic studies.

Methods

Field Experiment

The experiment was conducted in two locations: the Sawtooth Hatchery on the Salmon River near Stanley, Idaho, and the South Fork Salmon River (SFSR) satellite facility of the McCall Hatchery near Warm Lake, Idaho. Average water temperatures during the study were 10.9 °C at Sawtooth and 10.1 °C at SFSR. The carcasses used in this experiment were taken after normal hatchery spawning operations. Each carcass was tagged with a numbered disk. At Sawtooth Hatchery, we placed 12 male carcasses in a closed cage in the Salmon River. Fish lengths ranged from 56 cm to 79 cm fork length (FL, from tip of nose to fork of the caudal fin; mean = 69 cm). At SFSR, we suspended ten male carcasses in an unused raceway (FL 56 cm to 103 cm; mean = 84 cm), where they were held in water diverted from the river.

Carcasses were sampled every 2-3 days, beginning at the day of death until they fell apart (about 3 weeks). Hereafter, all references to carcass age and sample times are in days since death. At SFSR, the experiment ran from 30 August to 19 September. Samples were taken from each carcass every two days, resulting in a total of 11 samples per carcass. At Sawtooth Hatchery, the experiment ran from 1 September to 23 September. However, a nearby wildfire disrupted the sampling schedule at the beginning of the experiment. Samples were taken on day 0, day 3, day 6, and every other day thereafter for a total of 11 samples per carcass. Tissue samples were removed from caudal, anal, or pectoral fins using a hole punch. The punch was rinsed clean between samples. Samples were taken from areas of fin with the best color and condition, placed in 100% non-denatured ethanol, and stored at ambient temperature.

Laboratory Methods

Total genomic DNA was isolated from fin samples using a phenol-chloroform method (Paragamian et al. 1999). Recently, a collaborative effort developed a standardized set of 13 microsatellite loci for genetic stock identification of Chinook salmon across the Pacific Northwest (Seeb et al. 2007). We used the same 13 loci in this study: *Ots201b*, *Ots208b*, *Ots211*, *Ots212*, *Ogo4VIC*,

Ogo2, *Ots3M*, *Ots213*, *Omm1080*, *Ssa408*, *Ots9*, *OtsG474*, *Oki100*. PCR reaction conditions and thermocycler protocols are presented in Tables 1 and 2. All PCR products were electrophoresed using an ABI 3100 automated sequencer (Applied Biosystems) platform. Fragments were sized against GS500 LIZ size standard (Applied Biosystems) using GENEMAPPER 3.5.1 software (Applied Biosystems).

All samples were blindly scored by two technicians. Any sample that displayed more than 2 peaks, had a peak height of less than 200 intensity units, or did not conform to the usual morphology

of the locus were considered failures and were not scored. Samples that failed to amplify were not re-amplified. DNA concentration was quantified using a spectrophotometer (Eppendorf Biophotometer AG 22331 CE, Eppendorf, Germany).

Statistical Analyses

The purpose of the initial analysis was to determine which factors affect the probability of successfully obtaining the correct genotype. We used global logistic regression with location (Sawtooth or SFSR), locus, and time since death as class variables. The regression was implemented in SAS (Statistical

TABLE 1. PCR reaction conditions for each of the 13 microsatellite loci. For all reactions, 0.05 U/ μ l of Taq Polymerase and 1.0X Taq Buffer (Applied Biosystems) were used.

Locus	Reaction volume (μ l)	Extract (μ l)	MgCl ₂ (mM)	dNTPs (μ M)	Primers (μ M)	BSA (μ g/ μ l)
<i>Ogo4VIC</i>	15	1	1.8	0.8	0.6	0.8
<i>Ots211</i>	15	3	2.3	0.8	0.6	0.8
<i>Ots212</i>	15	1	1.8	0.8	0.3	0.8
<i>Omm1080</i>	15	3	2.0	0.8	0.8	1.0
<i>Ots213</i>	15	3	2.3	0.8	0.8	1.0
<i>Ots 3M</i>	15	2	1.8	0.8	1.0	0.0
<i>OtsG474</i>	15	1	2.0	0.6	0.8	0.8
<i>Ots 208b</i>	15	2	1.8	0.8	0.3	0.8
<i>Ogo2</i>	15	1	1.8	0.4	0.6	0.8
<i>Ots201b</i>	15	2	2.3	0.8	0.8	1.0
<i>Oki100</i>	10	3	2.5	0.8	0.8	1.0
<i>Ssa408</i>	10	3	2.3	0.8	0.6	0.8
<i>Ots9</i>	15	2	2.5	0.8	0.6	0.8

TABLE 2. Thermocycler protocols for each of the 13 microsatellite loci. Initial denaturation was at 95 °C for 2 min and cool-down was at 4 °C for 10 min. All reactions were run for 40 cycles.

Locus	Denaturation		Annealing		Extension		Final extension	
	Temp	Time	Temp	Time	Temp	Time	Temp	Time
<i>Ogo4VIC</i>	95	0:40	55	0:40	72	0:40	68	40:00
<i>Ots211</i>	94	0:40	60	0:40	72	0:40	72	40:00
<i>Ots212</i>	94	0:40	60	0:40	72	0:40	72	40:00
<i>Omm1080</i>	94	0:40	55	0:40	72	0:40	68	40:00
<i>Ots213</i>	94	0:40	60	0:40	72	0:40	72	40:00
<i>Ots3M</i>	94	0:40	55	0:40	72	0:40	68	40:00
<i>OtsG474</i>	94	0:40	58	0:40	72	0:40	68	40:00
<i>Ots208b</i>	94	0:30	58	0:30	72	0:30	68	40:00
<i>Ogo2</i>	94	0:40	58	0:40	72	0:40	68	40:00
<i>Ots201b</i>	94	0:30	58	0:30	72	0:30	68	40:00
<i>Oki100</i>	95	0:40	53	0:40	72	0:40	68	40:00
<i>Ssa408</i>	94	0:40	48	0:40	72	0:40	72	10:00
<i>Ots9</i>	94	0:40	62	0:40	72	0:40	68	10:00

Analysis Systems, Cary, North Carolina, USA) using the LOGISTIC procedure. Scores for each locus and sample were converted to 0 or 1, corresponding to failure or success, respectively. If there was a discrepancy in genotype assignment to an individual over time, we assumed that the first genotype in the series was correct. Because of mismatches between locations in the sampling schedule at the start of the experiment (caused by the fire near Sawtooth Hatchery), only data from 9 dates were used in this analysis (0, 6, 8, 10, 12, 14, 16, 18, and 20 days from death). Acceptable risk of a Type 1 error was set at 0.05.

A separate analysis was required to quantify the time effect for each locus. Sample quality degraded quickly and this was not estimable by the global analysis due to the missing cells that resulted from the wildfire. We estimated a logistic regression for each locus using time since death as a continuous variable. Data from all dates were included in this analysis. We used the resulting equations to compute time to 50% success (t_{50i}) for each locus i (1 through 13). To determine if the degradation rate was related to marker size, we computed the mean observed allele size (\bar{a}):

$$\bar{a}_i = \frac{\sum_{j=1}^{2n} a_{ijk}}{2n},$$

where a_{ijk} is the size in base pairs of the allele k (1 and 2) at locus i from carcass j , and n is the number of carcasses with at least 1 successful amplification of locus i . We then regressed t_{50i} versus \bar{a}_i to describe the effect of marker size on degradation rate.

Errors were defined as genotypes that did not agree with the first genotype in a series of samples from the same carcass. These were summed by carcass, date, and locus. We also reported successes as a percentage of the scored amplifications.

Results

Quality and quantity of DNA from the samples was highly variable. A total of 670 of the 3146 amplifications (21%) were

scored. In most cases, samples were not scored because the PCR amplification failed (73%). Most failures were either samples that did not amplify or could not be scored due to multiple or ambiguous peaks. Six percent of all samples were not scored because of multiple alleles. Success rates for samples taken on day 0 averaged 69% across all loci. Of the 22 carcasses, only 3 carcasses had all 13 loci genotyped on day 0 (mean = 9 loci). Of the 13 loci, none were successfully genotyped from all day-0 carcasses (mean = 15 carcasses). On day 2, mean success rate was 67% across all loci. Success then declined through time. On days 3 and 4, mean success rates were 47% and 48 %, respectively. By day 6, success rate had declined to 12% and fluctuated around 10% for the remainder of the study (range 7% - 13% for days 6 - 22). In general, the probability of success declined with time and declined sharply between days 4 and 6 (e.g., Figure 1).

The loci screened were quite variable in number and size of alleles. *OtsG474* displayed 2 alleles while *Omm1080* displayed 13 alleles (Table 3). *Ots9* had the smallest alleles ($\bar{a} = 109$ bp) while *Omm1080* had the largest alleles ($\bar{a} = 286$ bp). Our measure of success, predicted day at 50% success, ranged from negative values to over 10 days, depending on locus. Mean allele size at each locus was correlated negatively with predicted success ($F = 5.71, P = 0.04$; Figure 2).

We deemed 35 genotypes were in error (Table 3). Most errors were false alleles (60% of the errors) rather than allelic dropout (40% of the errors)

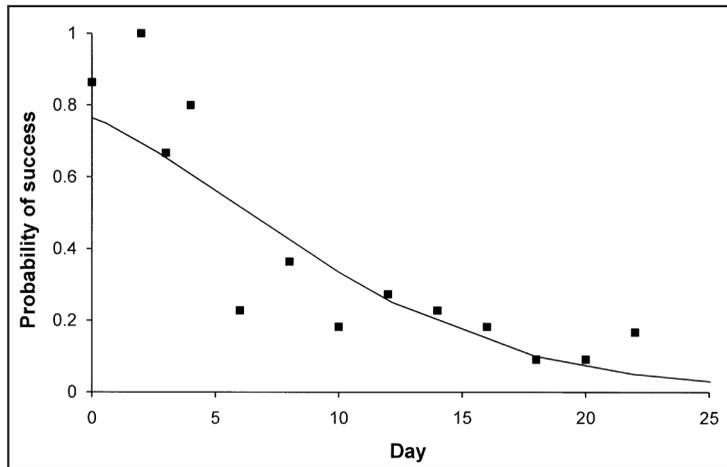


Figure 1. Decline in genetic quality through time for the *Ogo4VIC* locus. Observed data are the squares. The line is a fitted logistic regression.

TABLE 3. Number of alleles and mean allele size (bp, range in parentheses) observed in the 22 Chinook Salmon carcasses. Numbers of successful genotypes and errors also are presented.

Locus	Number of alleles	Allele size	Number of success/errors
<i>Ogo4VIC</i>	7	155 (142-162)	84/14
<i>Omm1080</i>	23	286 (218-350)	43/1
<i>Ots211</i>	17	274 (240-312)	36/0
<i>Ots212</i>	11	155 (135-207)	66/4
<i>Ots213</i>	14	265 (230-306)	31/0
<i>Ogo2</i>	6	219 (214-228)	13/0
<i>Ots3M</i>	4	146 (138-148)	21/0
<i>Ots201</i>	18	210 (165-302)	42/3
<i>Ots208</i>	19	217 (162-282)	45/4
<i>OtsG474</i>	2	157 (156-182)	118/0
<i>Oki100</i>	7	249 (212-287)	12/0
<i>Ssa408</i>	10	206 (188-232)	38/3
<i>Ots9</i>	5	109 (103-111)	96/6

and over half occurred within 2 loci (*Ogo4VIC*, *Ots9*). Errors first occurred on day 3 and their distribution was scattered through the rest of the experiment without obvious pattern. The global logistic regression was highly significant ($\chi^2 = 753.2$, $df = 21$, $P < 0.001$). The probability of obtaining a correct genotype was influenced by locus (Wald $\chi^2 = 224.7$, $df = 12$, $P < 0.001$), by time (Wald $\chi^2 = 412.9$, $df = 8$, $P < 0.001$), but not

by location (Sawtooth versus SFSR; Wald $\chi^2 = 0.3$, $df = 1$, $P = 0.59$). Sample DNA concentrations averaged 239 $\mu\text{g}/\mu\text{l}$ (range 5 - 1475 $\mu\text{g}/\mu\text{l}$). DNA concentrations were significantly related to time ($F = 17.19$, $P < 0.001$), however, the correlation was very imprecise ($r^2 = 0.07$) and did not add explanatory power over time to regression models of amplification success for 12 of 13 loci.

Discussion

Our main objective was to examine the probability of successful amplification through time with reference to costs and feasibility of NGS for salmon carcasses. Amplification success was influenced negatively by carcass age, and in as little as 4 days, amplification success rates dropped drastically. In this study, DNA degradation likely started before arrival to the spawning grounds and death. Success rates on day-0 carcass samples were lower than amplifications using fin clips from live juvenile Chinook salmon using the same set of loci (average = 89% across loci; M. Campbell, unpublished data). Adult Pacific salmon do not feed in freshwater and accomplish migration and spawning using stored energy reserves. Degenerative tissue changes can occur quickly in adult salmon once they enter freshwater; many spawning fish exhibit scars, abraded skin, deteriorated fins, and fungus on their bodies (Carruth et al. 2002, Gende et al. 2004). Fish returning to Idaho travel over 750 km and may be more spent metabolically at spawning time compared to coastal populations. Given this

long freshwater migration, it may even be difficult to extract DNA from fresh salmon carcasses in Idaho. There also may be a lot of variability in the amount of degradation in carcasses due to environmental conditions. Degradation in salmon carcasses is likely linked to temperature, moisture, bacteria, and fungi in the carcass, similar to studies investigating feces (Dallas et al. 2000, Piggot 2004, Murphy et al. 2007). We found no differences between study locations but water temperature and sampling procedures were similar at both hatcheries.

Success rates varied among loci and appeared to be related to amplicon size. The estimated time

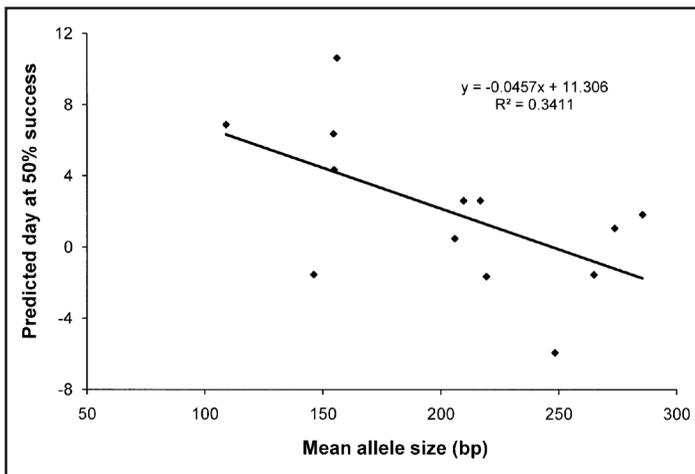


Figure 2. Relationship of marker size to rate of degradation at a locus. Marker size is the average allele size (bp). Predicted day at 50% success is used to index degradation rate.

to 50% success was negative for four loci, i.e., expected success rate was less than 50% at time of death. Larger loci were less likely to amplify than smaller ones, on average. This trend is characteristic of degraded DNA from non-invasive material (Wanderler et al. 2003, Martinkova and Searle 2006, Broquet et al. 2006). A genetic baseline for Chinook salmon in North America has been generated with the microsatellite loci used in this study (Seeb et al. 2007). Therefore, we cannot develop other microsatellite markers without compromising the ability to relate our data to other genetic studies in the Pacific Northwest. However, other genetic markers may be suitable for use on salmon carcass samples, given an appropriate scale of study objectives. For example, Schwenke et al. (2006) successfully differentiated among two Chinook salmon populations using single nucleotide polymorphism assays on degraded carcass tissue samples. Other microsatellites may show promise as well because amplification rates also can depend on the guanine-cytosine content and secondary structure of the amplicon (Lasken and Egholm 2003). Any prospective marker should be tested using carcass samples.

Genotyping errors were present in 5% of the scored samples and the majority of these errors were found in two loci with above average amplification rates (*Ogo4*, *Ots9*). This error rate was low compared to other NGS studies (Broquet et al. 2006, Valiere et al. 2007). We did not detect a relationship between the age of the carcass and error rates, but our amplification success rates were too low to be sensitive to any such correlation. Only 21% of the samples were scored, so the opportunity for investigation of genotyping errors was minimal. In our calculation of error rates, we assumed the first genotype in a series was correct because it was the freshest sample. In most cases, there was consistency of the first genotype with other samples in the time series where the error was observed (23 of 35 samples). Since most errors are not reproducible (Waits and Paetkau 2005), any genotype found more than once likely is the true genotype so we had additional confirmation that our sample was accurately scored in the first case. In six samples, only two genotypes were recorded; however, the first sample scored well at other loci while the error was found on a later date in a sample with fewer positive amplifications. In the remaining errors, the first sample was a heterozygote and the errors were homozygotes

indicating allelic drop-out. Undetected errors can result in inaccurate estimates of genetic diversity, population size, and parentage assessments but, as Neville et al. (2006b) demonstrated, carcass samples can yield useful information with appropriate replication.

An economical approach to obtaining genetic data from NGS is to develop screening techniques to identify and collect samples with high quality DNA (Morin et al. 2001, Hogan et al. 2008). When collecting tissues from salmon carcasses, field work should be conducted such that fresh carcasses are sampled. Since fresh samples cannot be accurately aged in the field, surveying streams every 1-3 days during the spawning season and marking fish already sampled will preclude the sampling of older carcasses. Also note gill color; all carcasses had lost gill color two days after death (J. Johnson, IDFG, unpublished data). In the laboratory, samples should be screened to determine DNA quality by amplifying a few of the largest loci and removing samples that failed to amplify. This approach may be most useful when carcass condition has not been noted, e.g., previously archived samples. Paetkau (2003) showed that this approach can increase success rates and decrease error rates as samples that failed at more than 50% of the loci had larger rates of allelic drop-out. Because there was no relationship between the amount of extracted DNA and amplification success, we do not recommend quantifying the DNA to determine the utility of a sample. A relatively large quantity of DNA may be measured but could be composed of small, degraded fragments (Taberlet and Luikart 1999). Other extraction and amplification procedures (e.g., preamplification) should be tested to optimize success rates for a particular set of loci. Lastly, even if screening procedures are used, independent replications of the PCR reactions are needed to reduce genotyping error rates (Taberlet et al. 1996). Given the error rates in our study, we recommend that simulations be performed at the onset of a study to determine the number of replications needed (Valiere et al. 2007). Otherwise, investigators should consider the biases potential errors may introduce into their studies.

Genetic information has become important in the study and management of Pacific salmon. Although the use of carcasses is not ideal, they can be a valuable source of genetic information when other types of samples are not available;

however, the level of effort needed to produce accurate and reliable data with microsatellite loci is considerable. Investigators need to incorporate this extra effort into project budgets and timelines. Not only will field collections need to be frequent to focus on fresh carcasses but data replication will also be necessary because of amplification failures and genotyping errors. Appropriate screening procedures may reduce some costs and new techniques and markers may give us the ability to analyze degraded tissue in a more cost-effective manner. However, regardless of the technique used, we recommend an evaluation of cost and time be performed at the onset of any study using salmon carcass tissues to evaluate population genetics so that useful data result from the effort invested.

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