DEVELOPMENT AND TESTING OF A SEMI-AUTOMATED MICROSATELLITE BASED GENOTYPING SYSTEM FOR KINSHIP ANALYSIS OF CHINOOK SALMON

FINAL REPORT

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ABSTRACT

Captive breeding is coming into increased use as a management tool for supplementation and restoration of depleted salmon populations. However, captive breeding is costly and can pose risks for the populations it is intended to help. Methods that would allow the relatedness of brood stock to be assessed and the parentage of offspring to be determined could increase the efficiency of captive breeding programs, facilitate monitoring of breeding outcomes, and reduce genetic risks to target populations. In this study a DNA-based method of determining relatedness (general relatedness coefficients and parent-offspring relationships) among individual chinook salmon was developed. A total of 64 microsatellite loci were screened, and used to select a panel of 14 highly variable loci for kinship determination. The panel of loci was tested using real chinook salmon families as well as simulated populations, and found to be highly effective for determining relatedness. Tests of the panel of loci on six chinook populations confirmed that the loci are sufficiently variable in all populations to serve in kinship analysis. The methods developed will permit relatively high throughput determination of relatedness in chinook salmon.

EXECUTIVE SUMMARY

The decline of many native west coast salmon populations has led to the drastic curtailment or elimination of once lucrative commercial fisheries. This loss of opportunity adversely effects those communities that rely on salmon as an important source of income. These extreme management restrictions place great emphasis on other restoration methods to assist and expedite the recovery of high-risk stocks. More over, harvest restrictions alone cannot be expected to enable the recovery of critically depressed populations. Hatchery supplementation is one method often used to aid in restoration efforts, however there are risks with this approach. Some of these risks are genetic and include loss of genetic variation through genetic drift and inbreeding, loss of genetic variation through outbreeding, and inadvertent selection as a result of hatchery practice (domestication selection).

In this project we evaluated a genetic tool, a microsatellite multiplex system, for evaluating genetic risks associated with hatchery supplementation of chinook salmon. The project had three objectives: 1) develop a high throughput multilocus genotyping system for high resolution kinship analysis and pedigree reconstruction; 2) develop a computer program(s) for inferring kinship and parentage from genetic data; 3) test the utility of this system on samples of chinook salmon from the Dungeness River Chinook Salmon Rebuilding Project (DRCSRP). The project consisted of two phases.

Phase 1 – Develop multilocus genotyping system

A 14 locus microsatellite multiplex system was developed that uses the polymerase chain reaction (PCR) to amplify microsatellites and an Applied Biosystems Inc. 373A fluorescent detection Sequencer/GeneScanner to visualize and size amplicons. The selection of loci was based on four criteria. The first two criteria were consistency and quality of amplification. Loci that amplified consistently and appeared as "sharp" bands on the 373A were chosen over loci that did not amplify, amplified inconsistently, and appeared as a diffuse and smeared band. Primer pairs for 16 loci were selected from a panel of 64 primer based on these criteria. The third and forth criteria were Mendelian inheritance and high polymorphism. The 16 loci were tested for Mendelian inheritance in three chinook salmon families. Fourteen

of the 16 loci exhibited allele segregation ratios consistent with Mendelian expectations. Two loci, because of null alleles, deviated significantly from Mendelian expectations and were discarded. Polymorphism of the remaining 14 loci were evaluated in six chinook salmon populations. The mean heterozygosity was about 0.80, the minimum defined in the project proposal, so all 14 loci were included in the multiplex system tested in phase 2.

Phase 2 – Test genotyping system on Dungeness river chinook salmon

A test of the 14 locus microsatellite multiplex system was conducted on captive brood stock from the Dungeness river chinook salmon restoration project to evaluate the system for kinship analysis and pedigree reconstruction. The multiplex system was used to evaluate two assumptions of relatedness of F_1 chinook salmon collected as juveniles from redds for captive brood stock: 1) F_1 chinook salmon from a single redd are full sibs; 2) F_1 chinook salmon from different redds are unrelated. The assumption of full sibship could not be rejected for F_1 chinook salmon from nine of 14 redds, suggesting these fish represented progeny from single pair matings. On the other hand the microsatellite multiplex system revealed that progeny from four redds represent multiple pair matings. Further, the assumption of no relatedness among individuals from different redds was rejected for four of seven redd pairs. The result of kinship analysis indicates the assumptions above are not valid for all redds and that resource managers should consider these genetic data when developing breeding schemes to avoid inbreeding and equalize founder contribution.

The multiplex system was also used to reconstruct a known two-generation pedigree. Two scenarios were considered: 1) a natural population with 2,500 candidate parent pairs; 2) a captive brood stock population with 134 candidate parent pairs. These scenarios reflect population sizes typically encountered in restoration programs. The results indicate that between four (captive brood stock scenario) and 10 (natural population scenario) of the 14 microsatellite loci will provide 95% parentage assignment success. These pedigree data will aid restoration managers in evaluating success of the restoration program in terms of maintaining genetic variability within the population. Finally, this microsatellite multiplex system should be useful in other populations of chinook salmon as indicated by the relative uniformity of heterozygosity estimates across loci in six different populations.

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1. PURPOSE

Description of problem

Declining salmon populations

The precipitous decline of many native west coast salmon populations has elevated public interest in stock conservation and rehabilitation efforts. As an example, the National Marine Fisheries Service (NMFS) has received petitions to list all populations of West Coast (OR, WA, ID, CA) chinook, and coho salmon and steelhead trout pursuant to the Endangered Species Act (ESA) (Steve Stone, NMFS, pers. com.). As of April 1, 1999 the NMFS has listed 24 distinct population segments (called evolutionarily significant units or ESU's) of six species (coho salmon, chinook salmon, chum salmon, sockeye salmon, steelhead trout, and coastal cutthroat trout) as either threatened or endangered under the ESA (Dandelski and Buck 1999). Much emphasis is being placed on rebuilding these depleted populations to levels that will permit once important fisheries to resume.

Initial efforts to rehabilitate salmon populations often include drastic curtailment or elimination of harvest on returning adults. This loss of opportunity adversely effects those communities that rely on harvesting salmon. Because salmon undergo long ocean migrations, the management implications of stock rehabilitation can be far reaching, resulting in complex and costly interstate and international negotiations (Huppert 1996). For example, in 1995 the catch quota for a lucrative commercial troll fishery in Southeast Alaska was reduced by federal court ruling to reduce incidental take of weak populations of chinook salmon bound for Canadian, Washington, Oregon and Idaho rivers (Huppert 1996). The loss of harvest opportunity and extreme management restrictions place great emphasis on other restoration methods to assist and expedite the recovery of high-risk stocks. Moreover, harvest restrictions alone cannot be expected to enable the recovery of critically depressed populations.

Hatchery supplementation

Hatchery supplementation is one component of recently developed chinook salmon rehabilitation projects (Waples et al. 1993; Hedrick et al. 1994; Smith and Wampler 1995;

USDE/BPA 1996). Supplementation differs from mitigation in that the former is intended to restore, not replace, depressed wild populations to self-sustaining levels and retain the genetic character of the wild population. When population numbers are extremely low captive broodstock programs may be used. In such programs populations are cultured in captivity throughout the entire life to improve survival of potential parents and ensure adequate breeding adults. In less extreme cases broodstock are taken from the target population, their progeny reared for a short time in the hatchery and then released into freshwater. Both methods improve survival, however their long-term effect on the genetic health of natural populations is unclear (Waples et al. 1993). Consequently, hatchery supplementation is the subject of research in four high profile chinook salmon restoration projects (Waples et al. 1993; Hedrick et al. 1994; Smith and Wampler 1995; USDE/BPA 1996).

Presumably, the genetic architecture of a population represents hundreds or thousands of years of adaptation to local conditions (Taylor 1991). Altering the genetic structure of the population through supplementation may nullify the effects of restoration and possibly put the population at greater risk. Genetic risks associated with supplementation include loss of within-population genetic variation through drift and inbreeding, loss of between-population genetic variation through outbreeding, and inadvertent selection as a result of hatchery practice (domestication selection) (Allendorf and Ryman 1987; Waples 1991; Waples 1993; Kapuscinski and Miller 1993; USDE/BPA 1996). Guidelines have been developed, based largely on theoretical considerations and some data, to minimize genetic risk associated with supplementation (e.g. Kapuscinski and Miller 1993). However, more empirical evidence is needed to assess the efficacy of supplementation with respect to maintaining genetic health of depressed populations.

Dungeness river chinook salmon

The Dungeness River Chinook Salmon Rebuilding Project (DRCSRP) is one example where hatchery supplementation is part of a salmon restoration program. This population is part of the Puget Sound ESU listed as threatened by the NMFS in February 1999. The goal of the DRCSRP is "to provide a self-sustaining, natural population that maintains the genetic characteristics of the existing chinook salmon stock and meets the agreed-to escapement goal three out of four years by the year 2008" (Smith and Wampler 1995). The centerpiece of this project, initiated in 1991, is a captive broodstock program. Progeny of 25 to 50 wild spawning adult pairs (the F_0 founder population) are taken from redds in river and isolated as single families in a hatchery. These first generation (F_1) individuals are reared in captivity until mature and artificially spawned. All crosses are made so as to avoid sibling mating. The second-generation (F_2) offspring are briefly reared in captivity before release into the Dungeness River.

To evaluate their success at equalizing founder contribution (Allendorf 1993) and maintaining the genetic characteristics of this population, the project supervisors need a tool for parentage analysis of the F_2 offspring. Further, they need a tool to verify first order relationships (e.g. full sibship) among F_1 adults to prevent inbreeding and avoid loss of genetic variation within the population.

<u>Kinship analysis and parentage assignment</u>

Existing marking technology is not capable of the fine scale genetic discrimination needed here. For example, physical tagging does not permit tracking genetic material across generations. Genetic tagging using protein coding loci (allozymes) does not allow evaluation of reproductive success of individual families, the ability to track family lineages across generations, or the ability to identify siblings to avoid inbreeding and assign parentage. These latter issues are of particular importance when restoring populations at very low number. Further, protein electrophoresis requires lethal sampling to acquire tissue which limits feasibility in threatened or endangered populations. In contrast, new techniques using DNA markers such as microsatellites are performed non-lethally and provide high resolution genetic discrimination (Bentzen et al. 1994; O'Reilly et al. 1996; Urquhart et al. 1995; Tessier et al. 1995).

Microsatellites are a class of nuclear DNA markers that are abundant in all eukaryotic genomes (Tautz 1989). They consist of 1-5 base pair (bp) repeating sequences that form arrays <300 bp in length, and exhibit high levels of co-dominant allelic variation in repeat number (Wright 1992; O'Reilly and Wright 1995). Polymorphism exhibited by specific microsatellites is readily detected by amplification of the microsatellite through the use of oligonucleotide primers specific to the non-repetitive regions that flank the repeat array, in combination with the polymerase chain reaction (PCR). Allelic variation is scored by gel electrophoresis of the PCR products, most commonly on denaturing acrylamide gels.

Microsatellites are presently used for gene mapping, forensics and parentage analysis in humans and other mammals (Edwards et al 1992: Ostrander et al 1993; Pepin et al; 1995; Urquhart et al. 1995). Microsatellites have begun to be applied in fisheries and aquacultural contexts, and display particular promise in high-resolution population and kinship studies (Wright and Bentzen 1994; McConnell et al. 1995; Nielson et al. 1994; O'Reilly and Wright 1995; Estoup et al. 1998). Nevertheless, the use of microsatellites for genetic research and monitoring of Pacific salmon is in its infancy. Technical development and empirical evaluation is needed to make best use of this powerful new genetic tool.

Project objectives

The goal of this project is to develop and test a system of multiplex microsatellite analysis for accurate, large-scale kinship analysis of chinook salmon. Such a system will permit critical evaluation of the success of chinook salmon restoration projects, and provide the tools needed to monitor pedigrees and avoid inbreeding in captive broodstock programs. The specific project objectives are as follows:

1. Develop a high throughput multilocus genotyping system for chinook salmon using microsatellite primer pairs previously screened and/or currently being developed in the Marine Molecular Biotechnology Laboratory (MMBL) in conjunction with 4-color fluorescent discrimination technology using the Applied Biosystems Inc. 373A automated sequencer/genescanner.
2. Develop program(s) for inferring kinship using the genetic data and a relational database.
3. Test the utility of this system on samples of chinook salmon from the Dungeness River Chinook Salmon Rebuilding Project (DRCSRP)

- Identify parents and grandparents of second generation hatchery-reared chinook salmon.
- Determine accuracy of kinship analysis.

2. APPROACH

Description of work

Phase 1 – Develop multilocus genotyping system

A. Screen microsatellites

Sixty four microsatellite loci were screened in chinook salmon using methods described by Olsen et al 1996. Screening consisted of amplification of each microsatellite locus via the Polymerase Chain Reaction (PCR). Primer pairs representing nine species of salmonid were tested for amplification effectiveness in 2 to 4 chinook salmon. Template DNA for PCR was isolated from 20-30 mg of fin tissue using procedures based on those for the Gentra Systems™ (Minneapolis MN) Puregene DNA isolation kit. PCR was carried out in 10 µL volumes (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.5 units *Taq* polymerase (Promega, Madison, WI), 0.3 µM each primer, and 100 ng DNA template) using a Perkin Elmer model 9600 thermo cycler. DNA amplifications generally involved the following profile: one cycle of 94°C (2 min); seven cycles of 94°C (1 min) + X°C (30 s) + 72°C (15 s); and 18 cycles of 94°C (30 s) + X°C (30 s) + 72°C (15 s) where X was an annealing temperature that varied among primer pairs. The results of each PCR were assessed using a Molecular Dynamics FluorImager™ 575 to detect fluorescently stained microsatellite alleles. Typically, 5 µL of each PCR product and 1 µL loading buffer (15% w/v ficoll 400, 0.06% w/v bromophenol blue, 0.06% w/v xylene cyanol, 30 mM EDTA) was loaded on a 20 cm, 6% non-denaturing polyacrylamide gel and electrophoresed for approximately 2 h at 150 V. At least two lanes of each gel contained 3 µL of Superladder-low 20 x 100 base pair (GenSura laboratories Inc.) size standard for estimating microsatellite allele length. Following electrophoresis the contents of each gel was stained with a 1:10,000 solution of SYBR™ Green 1 nucleic acid gel stain (Molecular Probes Inc.) and 1X Tris borate EDTA (TBE) buffer for 30 min and scanned on the FluorImager at a PMT voltage of 500-600.

B. Develop and test triplex PCRs and multiplex system

The six-locus multiplex described in the project proposal was used as a starting point for testing co-amplification and multiplexing of 16 loci selected during screening. Two loci (*One*µ14, *Ssa*85) of the original six locus multiplex were discarded because they exhibited excessive allelic stutter, making scoring difficult. The 16 loci were organized into two groups having PCR annealing temperatures of about 50°C and 59°C.

An ABI 373A semi-automated fluorescent detection system, in GeneScan™ mode, was used to test co-amplification and develop multiplexes (ABI 1993). The forward primer of each primer pair was labeled with one of three fluorescent labels. Label/locus combinations were selected based on locus allelic range to assure the greatest multiplexing potential (i.e. as many loci as possible in a single lane of an ABI 373A gel). We attempted co-amplification of various combinations of microsatellite primer pairs in each group starting first with those that provided the sharpest amplification product and were most polymorphic. Co-amplification was attempted in four individuals. Samples from each PCR were electrophoresed on the ABI373A using a 6% denaturing polyacrylamide gel to determine the quality of co-amplification. Approximately 1.0 µL of each PCR was combined with 3.15 µL formamide, 0.60 µL 50 mM EDTA and 0.25 µL (1.0 fmol) Perkin-Elmer GS500 internal size standard. All samples were denatured at 95°C for approximately 3 min, chilled on ice, and then loaded on the gel. Each gel was run for approximately 8 h at 25 W. Following the gel run, data were analyzed using the local Southern sizing algorithm in the GeneScan 672 analysis software, ver. 1.1 to estimate fragment length from the in lane standard (ABI 1993). Those groups in which all loci amplified were optimized by adjusting individual primer concentrations to equalize signal intensity as depicted by peak height on an electropherogram.

C. Verify Mendelian inheritance of candidate loci

Mendelian segregation was tested for the 16 candidate loci in three chinook salmon families using a chi-square test. A minimum of 30 offspring were genotyped per family to assure that expected cell values were always greater than 5.

D. Test multiplex system on six populations

The multiplex system was tested on six populations using the protocol described above. In addition, allele scoring and tabulation of data for importing into statistical software was performed with Genotyper software, ver. 2.0 (ABI 1996). Microsatellites *Oki*3a and *Ots*102 were excluded from the analysis because of null alleles. Expected heterozygosity was estimated for the remaining 14 loci using the equation

$$\hat{H}_E = 2n(1-\Sigma x_i^2)/(2n-1)$$

where n is the number of individuals in subpopulation X and x_i is the frequency of the ith allele (Nei 1987, pg. 178). The average $\hat{H}_E$ for each locus was calculated as the sum of $\hat{H}_E$ across populations divided by the number of populations. Tests for conformity to Hardy-Weinberg expectation (HWE) and genotypic linkage disequilibrium analyses were performed using a probability test in the computer program GENEPOP ver. 3.1b (Raymond and Rousset 1995). Statistical significance levels (α) for the probability tests were determined using sequential Bonferroni adjustments for simultaneous tests (Rice 1989).

<u>Phase 2 – Test genotyping system on Dungeness river chinook salmon</u>

E. Sample and genotype F_1 and F_2 individuals

As stated above, the founder population (F_0 generation) was allowed to spawn in the Dungeness river and their progeny (F_1 generation) were sampled as pre-emergent larvae from marked redds to establish the captive broodstock. In spring of 1993 larval chinook salmon were collected from 14 redds and were reared in freshwater to maturity (Smith and Wampler 1995). Samples of fin tissue were taken from mature F_1 adults in the fall of 1996 for DNA analysis. Tissue samples from F_2 juveniles (progeny of the 1996 F_1 matings) consisted of whole fish and were collected in the spring of 1997. All samples were preserved in 100% ethanol and stored in the laboratory at ambient temperature. The 14 locus multiplex system was used to genotype 147 F_1 and 100 F_2 chinook salmon.

F. Reconstruct F_0 allele pool and estimate relatedness among F_1 individuals

Genotype data from 147 F_1 chinook salmon (approximately 10 individuals per redd) were used to reconstruct the allele pool of each F_0 (founder) mating. The total number of alleles per locus was estimated for each redd as was the single locus genotypes for each founder (F_0) pair. Estimates of relatedness (r), within and among redds, were made for F_1 chinook salmon using the computer program RELATEDNESS ver. 5.0.1 (Goodnight and Queller 1997). RELATEDNESS is available on the World Wide Web at http://www-bioc.rice.edu/~kfg/GSoft.html. Confidence intervals for each estimate were made by jackknife sampling of loci. These data were used to test two assumptions of the captive broodstock program: 1) F_1 chinook salmon from a single redd are full sibs; 2) F_1 chinook salmon from different redds are unrelated.

G. Write computer database program

Two computer programs, CERVUS (Marshall et al. 1998) and PROBMAX (Danzmann 1998) were used to assign parentage of F_2 chinook salmon. CERVUS is available on the World Wide Web at http://helios.bto.ed.ac.uk/evolgen/ and PROBMAX is available by e-mail from the author at rdanzmann@uoguelph.ca. In addition, the simulation program PEDIGREE (Craig Busack, pers. com.) was used to evaluate the effect of full sibs of parents on pedigree reconstruction. These computer programs became available after this project began and eliminated the need to develop a database program.

H. Evaluate microsatellites for parentage analysis

CHINOOK SALMON PEDIGREE – Parentage analysis was performed on a known two-generation pedigree of chinook salmon from the Dungeness River captive broodstock program. Of the 147 F_1 adults genotyped for kinship analysis, 102 (48 males and 54 females) were used as broodstock in 1996. The mating scheme consisted primarily of 3x3 factorial crosses that did not include individuals from the same redd (putative full sibs). A total of 134 crosses (families) were made. One hundred parent pair-offspring relationships from 18 families (3-11 offspring per family) were subsampled for parentage analysis. The 18 families consisted of nine half sib pairs and represented the genetic contribution from all 14 redds (27 parents, Table

7). Between two and 11 full-sib relatives of each true parent were among the candidate parents (48 males and 54 females).

Fourteen microsatellite loci were used for parentage analysis (Table 8). Various measures of locus variability that indicate informative value for parentage analysis were computed. The average exclusion probability for a single unrelated parent-offspring pair was estimated for each locus (P_E) and for all loci ($P_E(C)$) using the computer program CERVUS. Other measures computed using CERVUS were locus heterozygosity (H_E) and polymorphic information content (PIC).

SIMULATED PEDIGREE – The simulation program PEDIGREE was used to evaluate the potentially confounding influence of full sibs of parents on pedigree reconstruction in the chinook salmon population. One hundred parent pair-offspring relationships were created from a population of unrelated candidate parents. Forty-eight male and 52 female genotypes were created from a random sample of a gamete pool generated from the chinook salmon allele frequency data. One hundred progeny genotypes were created by drawing a male and female parent at random and selecting one of two alleles at random from each locus from each parent. This process was repeated 1,000 times using 4, 6, 8, 10, 12, and 14 loci.

PARENTAGE ANALYSIS – Parentage analysis was conducted on both pedigrees using 4, 6, 8, 10, 12, and 14 loci included in descending order of P_E. Offspring were assigned parentage using exclusion and parent pair-offspring likelihood analysis. All possible crosses (2,592) were considered as candidate parent pairs. The computer program PROBMAX was used to identify non-excluded parent pairs for progeny in the chinook salmon pedigree. If multiple parent pairs were not excluded, then a parent pair-offspring (PPO) log-likelihood ratio (LOD) was computed for each non-excluded pair using the equation

$$\mathrm{LOD(QQ{:}UU)} = \sum_{l=1}^{L} \log_e[T_l(g_B|g_C,g_D)/P_l(g_B)]$$

where QQ:UU is the probability the parent pair-offspring trio are related versus the probability they are not related, g_B is the offspring genotype, g_C and g_D are the parental genotypes, T_l is the Mendelian segregation probability for the lth locus, and P_l is the genotype probability for the lth locus (Meagher and Thompson 1986). The offspring was counted as correctly assigned if the true parent pair had the highest PPO LOD score. Assignment success was defined as the percentage of offspring assigned to their true parent pair based on exclusion or PPO likelihood analysis. For the computer simulation this value was the mean from 1,000 pedigrees.

Offspring from the chinook salmon pedigree were also assigned parentage using single parent-offspring (SPO) likelihood analysis. An SPO LOD score was computed for all candidate parents of each gender using CERVUS. The male and female with the highest LOD scores were identified as the most likely parents and the offspring was counted as correctly assigned if they were the true parents.

The methods above considered all possible crosses since knowledge of the breeding pairs was not considered: common in studies of natural populations. Nevertheless, the breeding pairs in this study were known: common for most captive broodstock programs. PROBMAX was used to assign parentage given the limited pool of known matings (134). The results of this approach were compared to the results above that considered 2,592 (54 x 48) possible breeding pairs.

GENOTYPING PRECISION – Genotyping precision within and among gels was evaluated in two ways. First, one of four individuals from the adult sample was scored on every gel. If an allele was incorrectly scored at any locus for that individual, then the gel was rerun. Overall genotyping precision was measured for each locus and allele by calculating the standard deviation of fragment size estimates for each allele size category for each locus in Genotyper.

I. Estimate genetic variation and sample throughput

Three measures of genetic variation were computed (H_E, PIC, P_E) for each locus to assess their informative value for parentage analysis. An estimate of the efficiency of this genotyping system for pedigree reconstruction was computed for two scenarios: 1) the natural population scenario considered all possible crosses (2,592) as candidate parents; 2) the captive broodstock scenario considered only those crosses made in 1996 (134 crosses) as candidate parents. Efficiency was defined as the number of offspring typed in a 24 hour period using the number of loci required to achieve 95% assignment success. This definition assumes three

GeneScan runs per day (108 lanes) on the ABI 373A and all candidate parent genotypes are known.

Project management

This project was managed by the principle investigator, Dr. Paul Bentzen. Development and testing of microsatellites was conducted by Jeff Olsen with assistance from Jennifer Britt. Dr. James Shaklee of the Washington Department of Fish and Wildlife and his staff provided tissue samples for DNA analysis and provided the breeding records and redd origin of the chinook salmon captive broodstock.

3. FINDINGS

Accomplishments and findings

<u>Phase 1 – Develop multilocus genotyping system</u>

A. Screen microsatellites

The screening results are reported in Table 1. Forty seven of 64 microsatellites amplified in chinook salmon of which 16 were selected for multiplex development. Selection criteria included quality of amplification (i.e. loci with "sharp" bands were chosen over those that appeared as smears), consistency of amplification, and degree of polymorphism. Loci known to exhibit relatively high levels of polymorphism in chinook or other salmonids were selected in order to achieve a mean expected heterozygosity (H_E) of 0.80 to 0.90. Tetra-nucleotide repeat microsatellites were preferred because they tend to be more polymorphic than di-nucleotide repeat loci and the amplicons have fewer shadow bands or "stutter".

B. Develop and test triplex PCRs and multiplex system

Four groups of loci were chosen; two groups with an annealing temperature of 58°C and two groups with an annealing temperature of 50°C (Table 2). Due the large allelic range of some loci three lanes per individual were ultimately required (see Figure 1). Multiplex groups one and two required a separate lane of the ABI 373A gel while groups three and four were combined, post PCR, and loaded in a third lane. Two additions were made to the PCR profile reported above. First, a five step "touch down" was added to reduce the "noise" caused by amplification of non-target DNA. Second, a 30 minute extension cycle was added to promote amplification of adenylated fragments of di-nucleotide microsatellite (Magnuson et al. 1996).

C. Verify Mendelian inheritance of candidate loci

Multiple chi-square tests for Mendelian segregation resulted in two significant deviations at the $\alpha = 5\%$ level (famAB3/Ots4, $P = 0.01$; famAB3/Ots1, $P = 0.01$; Table 3). The tests were not significant when the α level was adjusted for 44 simultaneous tests (adjusted $\alpha = 0.001$).

A small number of samples possessed aberrant phenotypes. For example, 20 offspring from family W2 lacked an allele from one or both parents at microsatellite *Oki*3a. When both parents were assumed heterozygous with a single null allele (Callen et al. 1993), a Mendelian model of inheritance was not rejected ($P = 0.40$). Similar evidence of a null allele was also found for microsatellite *Ots*102 in one of 18 families used for parentage analysis (data not shown). Finally, five offspring possessed three alleles at one or more loci, and when they exhibited a two allele phenotype the electropherogram peak heights in Genotyper differed by a factor of about two, suggesting a three-dose genotype. These offspring, from family AB3 (4) and AA1 (1), apparently received two maternal alleles, consistent with spontaneous triploidy (e.g. Thorgaard and Gall 1979; Miller et al. 1994). Therefore these offspring were not included in the allelic segregation test.

Eleven progeny from family AB3 possessed alleles at locus *Ots*104 not present in their parents. The alleles (205 and 249) were observed one and ten times respectively. One explanation is a germline mutation. *Ots*104 is a tetranucleotide microsatellite and these alleles could represent a single repeat unit gain and loss at parental alleles 201 and 253. Alternatively, the progeny may be offspring of another parental pair. However, this is unlikely given the fact that AB3 parentage is confirmed at all other loci – a highly improbable result if the offspring belong to another family. Therefore, we included these progeny in the segregation ratio test at all loci except *Ots*104

D. Test multiplex system on six populations

The average $\hat{H}_E$ per locus ranged from 0.549 (*Ots*1) to 0.947 (*Ots*100) and the number of alleles per locus ranged from 10 (*Ots*1) to 57 (*Ots*100) (Table 4). The average $\hat{H}_E$ for multiple loci was 0.866 for the nine most polymorphic loci and 0.794 for all loci. Probability tests of Hardy-Weinberg expectation (HWE) at each locus showed 12 significant deviations at the $\alpha = 5\%$ level (Table 4). The tests were not significant, however, when the α level was adjusted for 84 simultaneous tests using the sequential Bonferroni procedure (adjusted α = 0.0006). Tests for genotypic linkage disequilibrium resulted in one significant *p*-value (*Ocl*1 x *Ogo*4 in population 1) when the α-level was adjusted to 0.0002 for 315 simultaneous tests.

<u>Phase 2 – Test genotyping system on Dungeness river chinook salmon</u>

E. Sample and genotype F_1 and F_2 individuals

See above (Approach – Task E)

F. Reconstruct F_0 allele pool and estimate relatedness among F_1 individuals

The total number of alleles per locus was estimated for each redd using 135 of the original 147 F_1 individuals (Table 5). Estimates of relatedness (r) indicated 12 F_1 individuals grouped more closely with individuals from redds different than their own. It is likely that the true redd identity for these 12 individuals were lost due to label mishandling. Thus, they were removed from the data set and further analysis was done using the remaining 135 individuals.

More than 4 alleles were found at one or more loci in four of 14 redds (9.0, 10.4, 15.2, 17.6b) indicating these progeny represent more than one parental pair (Table 5). The "extra" alleles in redd 17.6b were common to three individuals and no more than four alleles were found at any locus in the other seven individuals. Since these seven individuals appeared to be full sibs their genotypes were used to estimate the single locus parental genotypes reported for redd 17.6b. No such relationships were evident for individuals in redd 9.0, 10.4 and 15.2 and thus an estimate of their single locus parental genotypes could not be made. For the other 10 redds no more than four alleles were found indicating these F_1 progeny represent a minimum of one parental pair.

Single locus genotypes were estimated for each parental (F_0) pair (Table 6). Of the 11 redds for which parental (F_0) genotypes were estimated, four redd pairs possessed a common genotype at all loci (Table 6). The most likely explanation is these redd pairs share a common parent and the F_1 progeny are half sibs.

The hypothesis of full sibship could not be rejected for F_1 chinook salmon from nine of 14 redds. That is, the 95% confidence interval of the relatedness estimate included 0.5, the expected value for full sibs, while the lower limit was larger than zero, the expected value for unrelateds (Figure 2). The upper limit for the 95% confidence interval fell below 0.5 for redds 10.4 and 15.2 while the lower limit was greater than zero. In fact, the confidence intervals for redds 10.4 and 15.2 included an r of 0.25, the expected value for half sibs. This was consistent with the allele counts, which suggested multiple parental pairs contributed to these redds.

The upper limit for the 95% confidence interval fell below 0.5 for redds 4.3 and 6.2 but the lower limit was greater than 0.25. Conversely, the lower limit of the confidence interval was greater than 0.5 for redd 15.7. The basis for these results is still under investigation.

The hypothesis of no relatedness among redds was rejected for four redd pairs (Figure 3). These redd pairs (17.6a/17.6b, 15.9/15.7, 10.9/9.4, and 4.2a/4.2b) appeared to share a common parent (Table 6) and the 95% confidence intervals for the relatedness estimate included (or were near to) 0.25.

G. Write computer database program

See above (Approach – Task G)

H. Evaluate microsatellites for parentage analysis

PARENTAGE ANALYSIS – Estimates of expected heterozygosity (H_E) ranged from 0.553 (*Ots*1) to 0.946 (*Ots*100) and averaged 0.783 (Table 8). Estimates of PIC ranged from 0.450 (*Ots*1) to 0.932 (*Ots*100) and averaged 0.742. The average exclusion probability (P_E) for each locus for a single parent-offspring pair ranged from 0.152 (*Ots*1) to 0.768 (*Ots*100). The average exclusion probability for all loci, $P_E(C)$, exceeded 0.999.

Estimates of average relatedness among chinook salmon parents and their full-sib relatives ranged from 0.267 to 0.767 and averaged 0.467. All estimates were significantly greater than zero based on the 95% confidence interval generated from a jackknife sample of all loci.

The parentage assignment success was always lower for the chinook salmon pedigree than for the simulated pedigrees (Figure 4A). For example, the six most informative loci ($P_E(C) = 0.995$) provided a mean of 97% (SD = 1.91) unambiguous assignments for the simulations and 67% unambiguous assignments for the chinook salmon. The percentage of chinook salmon offspring with unambiguous parentage increased as loci were added but did not exceed 92% at 14 loci. Of the two likelihood methods, only PPO likelihood analysis increased assignment success for the chinook salmon (Figure 4A).

The mean number of non-excluded parent pairs (MPP) was always greater for the chinook salmon than for the simulations (Figure 4B). The mean estimate of pairwise relatedness (r) for non-excluded false parents and true parents in the chinook salmon exceeded 0.5 (the expectation for full sibs) when six or more loci were used for parentage analysis (Figure 4B).

The parentage assignment success varied between chinook salmon families (Table 9). Family AA1 always had more genetically compatible parent pairs than other families, including those families with a similar number of sampled progeny (AB3, W2). The mean of relatedness estimates for true parent/false parent pairs in family AA1 were always greater than 0.50 and were generally higher than in other families.

Finally, knowledge of the breeding pairs vastly improved assignment success in the chinook salmon population by reducing the number of possible parent pairs to 134. Assignment success for 100 progeny was 95% (4 loci), 97% (6 loci), 99% (8 loci), and 100% (10 or more loci). All assignments were unambiguous and PPO likelihood analysis did not resolve parentage in the few instances where multiple parent pairs were not excluded. Although nine pairs of half-sib families were sampled, in no instance were half sibs incorrectly assigned the same parent pair.

GENOTYPING PRECISION – The mean standard deviation of fragment size estimates in all allele size categories for each microsatellite ranged from 0.08 bases (*One*μ10) to 0.43 bases (*Ots*100) and was 0.19 bases over all loci (Table 8). Fragment sizing precision was highest for dinucleotide loci, with the exception of *Ocl*1. The lower sizing precision of tetranucleotide alleles did not effect genotyping accuracy because most alleles differed by four bases, allowing for non-contiguous allele categories.

I. Estimate genetic variation and sample throughput

Three measures of genetic variation that indicate informative value for parentage analysis (H_E, PIC, P_E) are reported in Table 8. Loci ranked the same according to informative value whether by H_E PIC, or P_E, with the exception of *Ocl*1 and *Ots*104.

An estimate of the efficiency of this genotyping system for pedigree reconstruction was computed for the two scenarios described above. Under the natural population scenario a minimum of 10 loci (2 lanes per individual) were required for 95% assignment success so it was possible to type 54 offspring (51 correct assignments) in 24 hours. Under the Dungeness River captive broodstock scenario a minimum of 4 loci (1 lane per individual) were required for 95% assignment success required so it was possible to type 108 offspring (102 correct assignments) in 24 hours.

Finally, it is important to point out that efficiency is defined here for an ABI 373A with 36 lanes. An upgrade is available for this machine that provides 64 lanes and would increase efficiency under the natural population scenario (2 lanes per individual) to 96 offspring per day (91 correct assignments). Using an ABI 377 could make further increases in efficiency. Electrophoresis on this machine is faster than the 373A – it is reasonable to expect six GeneScan runs in a 24 hour period. Depending upon the number of lanes (36, 64, 96), the ABI 377 would increase efficiency under the natural population scenario (10 loci) to 108 (102 correct assignments), 192 (182 correct assignments), or 288 (273 correct assignments).

Need for additional work

The 14 locus multiplex system described in this report is an effective tool for kinship and pedigree analysis of Dungeness River chinook salmon. Further, the multiplex system should be useful in other populations of chinook salmon as indicated by the relative uniformity of heterozygosity estimates across loci (Table 4). No additional development is necessary but some effort may be required to transfer this technology to agency labs responsible for genetic monitoring of restoration programs. The amount of effort required will depend upon the knowledge and expertise of the agency staff.

4. EVALUATION

Objectives versus results

This project was designed to meet three objectives. Each objective is reviewed here with respect to the project results described above.

1. Develop a high throughput multilocus genotyping system

PCR multiplex and fluorescent detection technology was used to create a multilocus genotyping system of 14 highly polymorphic microsatellites. This system proved to be effective for kinship analysis and pedigree reconstruction in chinook salmon. As shown above, genotyping efficiency for pedigree reconstruction will vary depending upon the instrument used (e.g. ABI 373A, ABI 377) and the size of the parental population. Under the natural population scenario 10 of the 14 loci were required for 95% assignment success and the genotyping throughput ranged from 54 offspring per day (ABI 373A with 36 lanes) to 288 offspring per day (ABI 377 with 96 lanes). Under the captive broodstock scenario just 4 of the 14 loci were required for 95% assignment success and the genotyping throughput ranged from 108 offspring per day to (ABI 373A with 36 lanes) to 576 offspring per day (ABI 377 with 96 lanes).

2. Develop program(s) for inferring kinship and parentage

No computer programs were developed. Instead, the computer programs RELATEDNESS, CERVUS, and PROBMAX were used to infer relatedness and assign parentage. These programs, which became available after the project was initiated, can be accessed through the World Wide Web or by e-mail from the author (see above). In addition, the simulation program PEDIGREE was used to evaluate the effect of full sibs of parents on pedigree reconstruction. This program was written by Dr. Craig Busack at the Washington Department of Fish and Wildlife to assist in development of genotyping systems for parentage analysis. Copies of the program PEDIGREE can be obtained by e-mail from Dr. Busack at (busaccsb@dfw.wa.gov).

3. Test the utility of this system on samples of chinook salmon

The 14 locus multiplex system was used to infer relatedness and parentage of chinook salmon from the Dungeness River captive brood stock program. In this case the true genealogies were known, or assumed, so this chinook salmon population provided a test of accuracy of the genotyping system for kinship and parentage analysis. The results indicate the microsatellites used here can be applied for fine scale kinship analysis to assist in restoration of the Dungeness River Chinook Salmon as well as other Chinook Salmon populations. For example, these microsatellites can be used to test assumptions of relatedness among groups of individuals used as brood stock. This test will aid resource managers in developing breeding schemes that avoid inbreeding and equalize founder contribution. Another important application is pedigree reconstruction. The results presented here indicate these microsatellites provide a high degree of parentage assignment success when applied to population sizes typically encountered in restoration programs. This pedigree data will aid restoration managers in evaluating success of the restoration program in terms of maintaining genetic variability within the population.

Dissemination of project results

In addition to the final report, this project will result in two manuscripts for scientific publication. The first manuscript titled "The aunt and uncle effect: an empirical evaluation of the confounding influence of full-sibs of parents on pedigree reconstruction" is complete and has been submitted to a peer reviewed journal. The second manuscript is in preparation and will describe kinship analysis of F_1 chinook salmon from the captive brood stock. Jeff Olsen also describes results of the pedigree analysis in chapter five of his Ph.D. dissertation. A copy of the dissertation is available at the University of Washington library and a copy of the abstract is available through author search on the World Wide Web at http://wwwlib.umi.com/dissertations/. Finally, genotype data for chinook salmon and the computer input files used in this study may be obtained by e-mail from Jeff Olsen at (jeff_olsen@fishgame.state.ak.us)

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7. TABLES AND FIGURES

Table 1. Microsatellite screening results for chinook. Loci used in multiplex development are shown in bold. The annealing temperature (°C) and amplification results – product observed (Y), product not observed (N) – are shown.

Locus	Source Species	Reference	Results
*Fgt*1	Rainbow trout	Sakamoto et al. 1993	56 N
***Ocl*1**	**Coastal cutthroat trout**	**Condrey and Bentzen 1998**	**60 Y**
*Ocl*2	"	"	60 Y
*Ocl*3	"	"	60 Y
*Ocl*4	"	"	55 Y
*Ocl*8	"	"	55 N
*Ocl*9	"	"	59 Y
*Ogo*1a	Pink salmon	Olsen et al. 1998	59 Y
*Ogo*1b	"	"	60 N
*Ogo*1c	"	"	60 N
***Ogo*2**	**"**	**"**	**58 Y**
*Ogo*3	"	"	59 Y
***Ogo*4**	**"**	**"**	**60 Y**
*Ogo*5	"	"	55 Y
*Ogo*6	"	"	60 Y
*Ogo*8	"	"	55 Y
***Oki*3a**	**Coho salmon**	**A. Spidle pers. com.**	**59 Y**
*Oki*4	"	"	50 N
*Oki*14	"	"	50 n
*Oki*19	"	"	50 Y
*Oki*20	"	"	50 Y
*Omy*77	Rainbow trout	Morris et al. 1996	50 Y
*Omy*78	"	M. O'Connel pers. com.	55 N
*Omy*87	"	"	55 N
*Omy*207	"	"	56 Y
*Omy*293	"	"	55 N
***Omy*325**	**"**	**"**	**58 Y**
*One*µ1	Sockeye salmon	Scribner et al. 1996	58 N
*One*µ2	"	"	58 N
*One*µ4	"	"	58 Y
***One*µ8**	**"**	**"**	**58 Y**
*One*µ9	"	"	58 Y
*One*µ10	"	"	57 Y
*One*µ11	"	"	58 Y
*One*µ14	"	"	58 Y
***Ots*1**	**Chinook salmon**	**Banks et al. 1999**	**50 Y**
***Ots*2**	**"**	**"**	**48 Y**
***Ots*3**	**"**	**"**	**50 Y**
***Ots*4**	**"**	**"**	**56 Y**

Table 1. cont.

Locus	Source Species	Reference	Results
*Ots*5	Chinook salmon	Banks et al. 1999	45 Y
*Ots*6	"	"	57 Y
***Ots*100**	"	**Nelson and Beacham 1999**	**59 Y**
***Ots*101**	"	**Small et al. 1998**	**50 Y**
***Ots*102**	"	**Nelson and Beacham 1999**	**50 Y**
*Ots*103	"	Beacham et al. 1998	58 Y
***Ots*104**	"	**Nelson and Beacham 1999**	**50 Y**
*Ots*105	"	"	52 Y
*Ots*106	"	"	52 Y
***Ots*108**	"	"	**50 Y**
*Sfo*8	Brook trout	Angers et al. 1995	60 Y
*Sfo*12	"	"	50 N
*Sfo*18	"	"	52 N
*Sfo*23	"	"	52 N
*Ssa*4	Atlantic salmon	McConnell et al. 1995	57 Y
*Ssa*14	"	"	52 Y
*Ssa*85	"	O'Reilly et al. 1996	58 Y
*Ssa*171	"	"	56 Y
*Ssa*197	"	"	57 Y
*Ssa*202	"	"	58 N
*Ssa*289	"	"	46 N
*Ssa*293	"	M. O'Connel pers. com.	53 Y
μ*Sat*15	Brown trout	Estoup et al. 1993	57 N
μ*Sat*60	"	"	60 Y
μ*Sat*73	"	"	57 Y

Table 2. Microsatellite multiplex sets developed for kinship analysis in chinook salmon. The PCR annealing temperature is shown in bold.

Group	Locus	Repeat	Dye label	MgCl (mM)	Primer (uM)	PCR Profile
1	*Oki*3a	tetra-	6fam	2.0	0.070	5x(94(1min)+**63 to 59(30sec)TD**+72(15sec))
	*One*µ8	di-	tet		0.080	7x(94(1min)+**58(30sec)**+72(15sec))
	*Ocl*1	di-	hex		0.050	17x(94(30sec)+**58(30sec)**+72(15sec))
	*Omy*325	di-	tet		0.070	72(30 min)
	*Ots*100	tetra-	tet		0.090	4(hold)
2	*Ots*101	tetra-	6fam	2.0	0.180	5x(94(1min)+**55 to 51(30sec)TD**+72(15sec))
	*Ots*102	tetra-	tet		0.180	7x(94(1min)+**50(30sec)**+72(15sec))
	*Ots*104	tetra-	hex		0.180	17x(94(30sec)+**50(30sec)**+72(15sec))
	*Ots*2	di-	6fam		0.350	72(30 min)
	*Ots*3	di-	tet		0.350	4(hold)
	*One*µ10	di-	hex		0.280	
3	*Ogo*4	di-	hex	2.5	0.120	same as group 1
	*Ots*4	di-	6fam		0.050	
	*Ogo*2	di-	6fam		0.180	
4	*Ots*1	di-	6fam	1.5	0.250	same as group 2
	*Ots*108	tetra-	tet		0.150	

Table 3. Inheritance of 16 microsatellite loci in three Chinook salmon families.

Family	*Ocl*1	obs	exp	*Oki*3A	obs	exp	*Omy*325	obs	exp	*One*μ8	obs	exp	*Ots*100	obs	exp	*One*μ10	obs	exp
AB3																		
female	162/168			152/223			91/91			167/183			270/314			138/144		
male	168/174			195/215			91/91			177/177			270/320			144/150		
offspring	162/174	18	23.25	152/195	23	23.25	91/91	93	93	167/177	44	46.5	270/270	26	23	138/144	27	23.25
	162/168	31	23.25	152/215	23	23.25				177/183	49	46.5	270/314	31	23	138/150	26	23.25
	168/168	20	23.25	195/223	28	23.25							270/320	17	23	144/144	14	23.25
	168/174	24	23.25	215/223	19	23.25							314/320	18	23	144/150	26	23.25
Total		93			93			93			93			92			93	
X^2 *p*-value		0.24			0.63			1.00			0.60			0.12			0.18	
W2																		
female	161/169			151/null			91/91			177/179			226/310			138/140		
male	161/163			223/null			99/123			161/173			290//346			144/144		
offspring	161/161	9	7.75	151/223	11	7.75	91/99	16	15.5	161/177	7	7.75	226/290	12	7.75	138/144	16	17
	161/163	7	7.75	151/null	6	7.75	91/123	15	15.5	173/177	5	7.75	226/346	10	7.75	140/144	18	17
	161/169	8	7.75	223/null	9	7.75				161/179	11	7.75	290/310	3	7.75			
	163/169	7	7.75	null/null	5	7.75				173/179	8	7.75	310/346	6	7.75			
Total		31			31			31			31			31			34	
X^2 *p*-value		0.95			0.40			0.86			0.49			0.10			0.73	
AA1																		
female	159/163			219/223			91/91			177/177			262/274			140/144		
male	161/163			175/195			123/123			175/177			274/274			144/150		
offspring	159/161	5	8	175/219	10	8	91/123	32	32	175/177	19	16	262/274	18	16	140/144	9	7.75
	161/163	8	8	175/223	8	8				177/177	13	16	274/274	14	16	140/150	10	7.75
	163/163	7	8	195/219	8	8										144/144	8	7.75
	159/163	12	8	195/223	6	8										144/150	4	7.75
Total		32			32			32			32			32			31	
X^2 *p*-value		0.35			0.80			1.00			0.29			0.48			0.44	

Table 3. cont.

Family	*Ots*104	obs	exp	*Ots*101	obs	exp	*Ots*2	obs	exp	*Ots*102	obs	exp	*Ots*3	obs	exp	*Ogo*2	obs	exp
AB3																		
female	253/263			183/191			69/87			223/328			87/91			224/226		
male	201/201			183/215			85/105			340/340			91/95			220/224		
offspring	201/253	35	41	183/183	14	23	69/85	21	22.75	223/340	39	46.5	87/91	23	22.75	220/224	24	22.75
	201/263	47	41	183/215	24	23	69/105	25	22.75	328/340	54	46.5	87/95	30	22.75	224/224	23	22.75
				183/191	30	23	85/87	18	22.75				91/91	24	22.75	220/226	26	22.75
				191/215	24	23	87/105	27	22.75				91/95	14	22.75	224/226	18	22.75
Total		82			92			91			93			91			91	
X^2 *p*-value		0.19			0.13			0.54			0.12			0.12			0.68	
W2																		
female	203/223			259/259			201/247			87/93			91/95			224/226		
male	195/219			227/345			201/201			87/103			93/95			224/262		
offspring	195/203	8	8	227/259	19	16.5	201/201	19	16.5	87/87	6	8.25	91/93	9	8.25	224/224	7	8.5
	203/219	7	8	259/345	14	16.5	201/247	14	16.5	87/103	11	8.25	91/95	7	8.25	224/262	12	8.5
	195/223	9	8							87/93	7	8.25	93/95	6	8.25	224/226	7	8.5
	219/223	8	8							93/103	9	8.25	95/95	11	8.25	226/262	8	8.5
Total		32			33			33			33			33			34	
X^2 *p*-value		0.97			0.38			0.38			0.62			0.62			0.57	
AA1																		
female	210/221			195/215			83/93			255/279			95/97			224/226		
male	243/247			215/215			69/83			255/263			93/93			224/224		
offspring	210/243	8	7.75	195/215	12	15.5	69/83	7	7.5	255/255	8	7.5	93/95	14	13.5	224/224	19	15.5
	210/247	4	7.75	215/215	19	15.5	83/83	9	7.5	255/263	11	7.5	93/97	13	13.5	224/226	12	15.5
	221/243	11	7.75				69/93	9	7.5	255/279	7	7.5						
	221/247	8	7.75				83/93	5	7.5	263/279	4	7.5						
Total		31			31			30			30			27			31	
X^2 *p*-value		0.36			0.21			0.69			0.34			0.85			0.21	

Table 3. cont.

Family	*Ots*1	obs	exp	*Ots*4	obs	exp	*Ogo*4	obs	exp	*Ots*108	obs	exp
AB3												
female	184/194			148/148			136/136			108/120		
male	194/194			144/148			164/164			170/170		
offspring	184/194	56	43.5	144/148	58	45.5	136/164	91	22.75	108/170	44	45
	194/194	31	43.5	148/148	33	45.5				120/170	46	45
Total		87			91			91			90	
X^2 *p*-value		0.01			0.01			1.00			0.83	
W2												
female	188/194			144/148			136/154			158/174		
male	184/184			144/148			142/162			108/108		
offspring	184/188	14	16	144/144	9	11	136/142	7	8.25	108/158	15	16
	184/194	18	16	144/148	13	11	136/162	11	8.25	108/174	17	16
				148/148	11	11	142/154	7	8.25			
							154/162	8	8.25			
Total		32			33			33			32	
X^2 *p*-value		0.48			0.70			0.73			0.72	
AA1												
female	184/184			148/148			166/170			108/108		
male	184/194			148/148			136/164			174/178		
offspring	184/184	12	15.5	148/148	31	31	136/166	9	7.5	108/174	18	14.5
	184/194	19	15.5				164/166	5	7.5	108/178	11	14.5
							136/170	7	7.5			
							164/170	9	7.5			
Total		31			31			30			29	
X^2 *p*-value		0.21			1.00			0.69			0.19	

Table 4. Expected heterozygosity at 14 microsatellite loci in six Chinook salmon populations – Sandy River, Oregon (Pop1); Clackamas Hatchery, Oregon (Pop2); Yakima River, Washington (Pop3); Dungeness River, Washington (Pop4); Washougal River, Washington (Pop5); Elwha River, Washington (Pop6). Significant departures from HWE are marked with an asterisk (* = $P < 0.05$; ** = $P < 0.01$).

		Expected Heterozygosity						
		Pop1	Pop2	Pop3	Pop4	Pop5	Pop6	
Locus	A	n = 40	n = 40	n = 50	n = 45	n = 52	n = 46	Avg.
Ots100	57	0.939	0.952	0.961	0.946	0.954	0.931	0.947
Ots101	35	0.909	0.933	0.942	0.892	0.962	0.914	0.925
Ots104	46	0.946*	0.918*	0.934	0.845*	0.964**	0.937	0.924
Ots108	36	0.886	0.776	0.935	0.735	0.947	0.777	0.843
Ots2	17	0.841	0.822	0.693	0.870	0.835*	0.843	0.817
Oneu8	19	0.743	0.744	0.841*	0.828**	0.854	0.773	0.797
Ogo2	16	0.823	0.824	0.827	0.765	0.805	0.685	0.788
Omy325	15	0.805	0.751*	0.829	0.767	0.758	0.810	0.787
Ogo4	15	0.727	0.748	0.846	0.796	0.813	0.787	0.786
Avg.	28.4	0.846	0.830	0.868	0.827	0.877	0.829	0.846
Ocl1	12	0.768	0.703	0.677	0.847	0.827	0.835	0.776
Ots3	11	0.782*	0.748	0.595*	0.728	0.861	0.743*	0.743
Ots4	12	0.748	0.799	0.765	0.655	0.721	0.661	0.725
Oneu10	12	0.768	0.680	0.639	0.734	0.699	0.743*	0.710
Ots1	10	0.460	0.408	0.625	0.553	0.613	0.637	0.549
All avg.	22.4	0.796	0.772	0.794	0.783	0.830	0.791	0.794

Table 5. Number of alleles per redd at 14 microsatellite loci in 1992 Dungeness River Chinook salmon. Redds with one or more loci with five or more alleles are underlined.

Redd	n	Ocl1	Omy325	Oneu8	Ots100	Oneu10	Ots104	Ots101	Ots2	Ots3	Ogo2	Ogo4	Ots108	Ots4	Ots1
17.6a	10	4	3	2	4	3	2	2	2	3	3	3	3	3	2
<u>17.6b</u>	10	3	2	2	**5**	3	3	3	4	2	3	2	2	3	1
17.4	9	3	3	2	4	2	2	4	3	3	2	2	2	1	2
15.9	11	3	2	2	3	3	4	3	4	3	2	3	3	2	2
15.7	9	3	3	2	2	3	3	2	4	3	2	3	4	1	2
<u>15.2</u>	9	**5**	4	**5**	4	4	4	**5**	**6**	3	3	**5**	3	3	2
10.9	10	4	4	4	4	2	3	4	4	3	4	3	1	3	3
<u>10.4</u>	8	**6**	4	4	**6**	3	**6**	**5**	**5**	3	3	3	3	3	4
9.4	9	4	4	4	4	3	3	3	4	2	4	4	2	3	2
<u>9.0</u>	7	4	4	**7**	**5**	2	**5**	4	4	4	3	3	3	3	3
6.2	11	4	2	4	4	3	4	4	3	4	3	3	3	3	2
4.3	10	3	3	3	4	3	4	4	4	3	4	4	4	3	2
4.2a	12	3	3	4	3	3	4	4	4	2	2	3	4	2	1
4.2b	10	3	1	4	4	3	4	4	4	4	3	2	4	2	2

Table 6. An estimate of parental (F_0) single locus genotypes for 1992 Dungeness river Chinook salmon. Genotypes were reconstructed from offspring (F_1) data. Rectangles indicate redd pairs sharing a single genotype. The redd identity indicates distance (in miles) above the river mouth. Redds less than 0.1 miles apart are labeled as a or b. Note: redds 9.0, 10.4, and 15.2 are not shown because more than 4 alleles were found among progeny (see text).

Redd	Ocl1		Oki3a		Omy325		Onem8		Ots100		Onem10		Ots104		Ots101		Ots2		Ots102		Ots3		Ogo2		Ots1		Ots4		Ogo4		Ots108	
17.6a	165	169	195	195	99	91	185	177	274	262	144	144	201	217	215	183	85	85	255	215	93	95	220	224	184	194	148	150	148	164	108	170
	161	175	207	195	91	95	177	177	310	320	138	150	201	201	215	215	105	105	259	339	91	95	226	220	194	194	148	144	164	142	174	108
17.6b	169	169	195	195	91	91	177	177	270	262	144	144	217	217	183	183	91	85	215	215	95	95	224	224	194	194	148	150	164	164	170	170
	169	175	215	195	91	95	161	177	262	320	138	150	201	201	183	215	85	105	339	339	91	95	224	220	194	194	148	144	142	142	108	108
17.4	161	167	199	199	95	105	175	177	302	310	138	144	201	229	215	233	69	103	263	265	91	93	224	226	184	184	148	148	136	166	108	108
	175	175	215	215	91	95	177	175	270	274	144	144	229	229	223	241	85	85	265	255	95	93	224	224	194	194	148	148	136	136	108	112
15.9	159	163	223	195	91	91	175	175	262	270	140	144	201	221	195	215	93	83	279	255	97	95	226	224	184	194	144	148	136	170	112	178
	161	161	223	219	123	123	177	177	270	274	150	144	249	247	191	191	71	103	255	263	93	93	226	224	184	184	148	148	166	136	108	108
15.7	163	163	219	195	95	91	177	175	274	270	138	144	221	221	215	215	105	83	275	255	93	93	224	224	194	194	148	148	164	170	108	178
	157	161	null	219	123	123	177	177	274	274	150	144	243	247	215	191	69	103	255	263	91	95	226	224	194	184	148	148	136	136	174	128
10.9	163	175	199	151	91	99	173	185	278	346	150	150	247	201	195	223	91	87	259	345	93	95	220	230	184	194	148	148	148	142	108	108
	167	161	203	null	123	95	175	161	286	302	150	144	213	213	187	219	83	69	279	255	91	93	228	262	188	184	144	152	164	164	108	108
9.4	169	175	223	151	123	99	165	185	290	346	144	150	247	201	195	223	91	87	227	345	93	95	220	230	184	194	144	148	166	142	108	108
	163	161	null	null	145	95	173	161	338	302	138	144	201	213	195	219	103	69	279	255	93	93	224	262	194	184	144	152	162	164	174	108
6.2	167	169	151	227	91	95	167	177	278	314	144	144	217	293	183	249	69	73	259	275	91	95	224	240	194	194	144	148	142	148	112	116
	165	163	195	199	91	91	169	183	338	326	136	138	251	229	223	219	85	69	223	239	93	87	226	224	194	184	148	150	136	142	108	112
4.3	161	169	151	195	89	123	177	177	306	314	138	140	201	239	223	227	69	85	223	287	93	93	226	230	194	194	144	152	136	154	128	158
	161	175	null	null	91	91	173	179	310	226	140	150	231	247	237	203	93	87	259	259	91	95	220	224	188	194	152	148	164	142	174	108
4.2a	165	165	167	223	95	91	165	175	322	270	138	144	249	263	183	191	103	87	259	327	91	97	220	220	184	184	144	148	148	136	128	120
	179	169	175	223	89	91	177	183	306	306	144	152	247	231	237	175	69	85	265	227	97	91	226	226	184	184	148	148	136	142	140	174
4.2b	169	165	195	223	91	91	167	175	314	270	138	144	251	263	183	191	69	87	223	327	87	97	224	220	194	184	148	148	136	136	108	120
	163	169	151	223	91	91	169	183	326	306	144	152	217	231	223	175	73	85	259	227	95	91	226	226	194	184	150	148	142	142	112	174

Table 7. Eighteen chinook salmon families sampled for this study. The redd identity is provided for each parent which indicates distance (in miles) above river mouth. Redds less than 0.1 miles apart are labeled as a or b. The number of offspring typed for parentage analyses are shown in column PA.

Family	female	redd	male	redd	PA
AA1	F191	15.9	M183	15.7	11
AA2	F191	15.9	M192	10.4	5
AB2	F194	4.2b	M192	10.4	5
AB3	F194	4.2b	M198	17.6b	10
AD3	F215	6.2	M221	17.6b	4
AE1	F216	17.4	M218	10.9	5
AI1	F254	4.2b	M253	17.4	5
AK1	F258	6.2	M253	17.4	4
AM1	F292	4.2a	M281	15.9	5
AM2	F292	4.2a	M299	9.0	5
G1	F20	15.9	M23	10.9	4
G2	F20	15.9	M24	9.4	3
M1	F61	15.9	M61	4.2b	5
M2	F61	15.9	M62	15.2	5
T1	F98	10.9	M96	15.7	5
T2	F98	10.9	M97	17.6a	5
W1	F146	4.3	M152	15.7	4
W2	F146	4.3	M159	9.4	10

Table 8. Fourteen microsatellite loci used for parentage analysis in chinook salmon. Abbreviations indicate tetranucleotide (T), dinucleotide (D), PCR annealing temperature ($T°_m$), PCR multiplex group (a, b), number of alleles (A), allele range in bases (R), mean standard deviation of fragment size estimates (in bases) in all allele categories (MSD), expected heterozygosity (H_E), polymorphic information content (PIC), and average exclusion probabilities (P_E) for a single unrelated parent-offspring pair.

Locus		Reference	$T°_m$	A	R	MSD	H_E	PIC	P_E
*Ots*100	T	Nelson and Beacham1999	58a	19	214-402	0.43	0.946	0.932	0.768
*Ots*101	T	Small et al. 1998	50a	17	147-281	0.36	0.892	0.870	0.618
*Ots*2	D	Banks et al. 1999 in press	50a	10	69-105	0.12	0.870	0.846	0.565
*Ots*104	T	Nelson and Beacham1999	50a	14	157-323	0.39	0.845	0.820	0.523
*Ocl*1	D	Condrey and Bentzen 1998	58a	10	149-179	0.22	0.847	0.817	0.504
*One*μ8	D	Scribner et al. 1996	58a	11	157-191	0.15	0.828	0.801	0.488
*Ogo*4	D	Olsen et al. 1998	58b	8	136-184	0.13	0.796	0.757	0.411
*Omy*325	D	O'Connell (pers. comm.)	58a	8	85-145	0.14	0.767	0.730	0.380
*Ogo*2	D	Olsen et al. 1998	58b	7	210-262	0.14	0.765	0.719	0.360
*Ots*108	T	Nelson and Beacham1999	50b	13	100-298	0.16	0.735	0.705	0.356
*One*μ10	D	Scribner et al. 1996	50a	6	134-156	0.08	0.734	0.679	0.312
*Ots*3	D	Banks et al. 1999	50a	5	85-105	0.15	0.728	0.670	0.299
*Ots*4	D	Banks et al. 1999	58b	5	140-162	0.10	0.655	0.594	0.235
*Ots*1	D	Banks et al. 1999	50b	5	178-196	0.15	0.553	0.450	0.152
mean				9.86		0.19	0.783	0.742	
$P_E(C)$									0.999

Table 9. Parentage assignment success (as), number of genetically compatible parent pairs (pp), and mean of relatedness estimates (r) for all true parent/false parent pairs for (n) offspring from 18 chinook salmon families. The number of related candidate parents are shown for each female (R_F) and male (R_M) parent.

						14 loci			12 loci			10 loci			8 loci			6 loci			4 loci		
fam	♀	R_F	♂	R_M	n	as	pp	r	as	pp	r	as	pp	r	as	pp	r	as	pp	r	as	pp	r
AA1	191	10	183	4	11	11	4	0.69	10	4	0.69	10	7	0.67	10	6	0.67	5	12	0.60	3	21	0.56
AA2	191	10	192	2	5	5	1		5	1		5	3	0.64	5	3	0.64	4	3	0.64	1	11	0.21
AB2	194	6	192	2	5	5	1		5	1		5	1		5	1		5	1		0	13	0.24
AB3	194	6	198	5	10	10	2	0.66	10	2	0.66	10	2	0.66	10	3	0.56	5	5	0.59	2	13	0.30
AD3	215	9	221	5	4	4	1		4	1		4	1		4	1		4	1		1	6	0.48
AE1	216	6	218	9	5	5	1		5	1		5	1		5	1		3	2	0.35	0	13	0.17
AI1	254	6	253	6	5	5	1		5	1		5	1		5	1		5	2	0.29	50	3	0.47
AK1	258	9	253	6	4	4	1		4	1		4	1		4	1		4	3	0.56	3	6	0.47
AM1	292	11	281	10	5	5	1		5	1		5	1		5	1		5	1		3	8	0.53
AM2	292	11	299	4	5	5	1		5	1		5	1		4	4	0.63	4	4	0.63	3	6	0.57
G1	20	10	23	9	4	4	1		4	1		4	1		4	1		4	1		0	8	0.35
G2	20	10	24	7	3	2	2	0.67	2	2	0.67	2	2	0.67	1	4	0.51	1	4	0.51	0	8	0.49
M1	61	10	61	6	5	5	1		5	1		5	1		5	1		5	1		3	3	0.19
M2	61	10	62	6	5	5	1		5	1		5	1		5	1		5	1		4	3	0.39
T1	98	9	96	4	5	5	1		5	1		5	1		5	1		5	4	0.59	4	10	0.33
T2	98	9	97	5	5	5	1		5	1		5	1		4	2	0.66	3	3	0.62	2	7	0.51
W1	146	3	152	4	4	4	1		3	2	0.66	3	2	0.66	2	4	0.71	0	5	0.60	2	10	0.32
W2	146	3	159	7	10	10	1		10	1		10	1		10	1		9	3	0.44	7	4	0.29

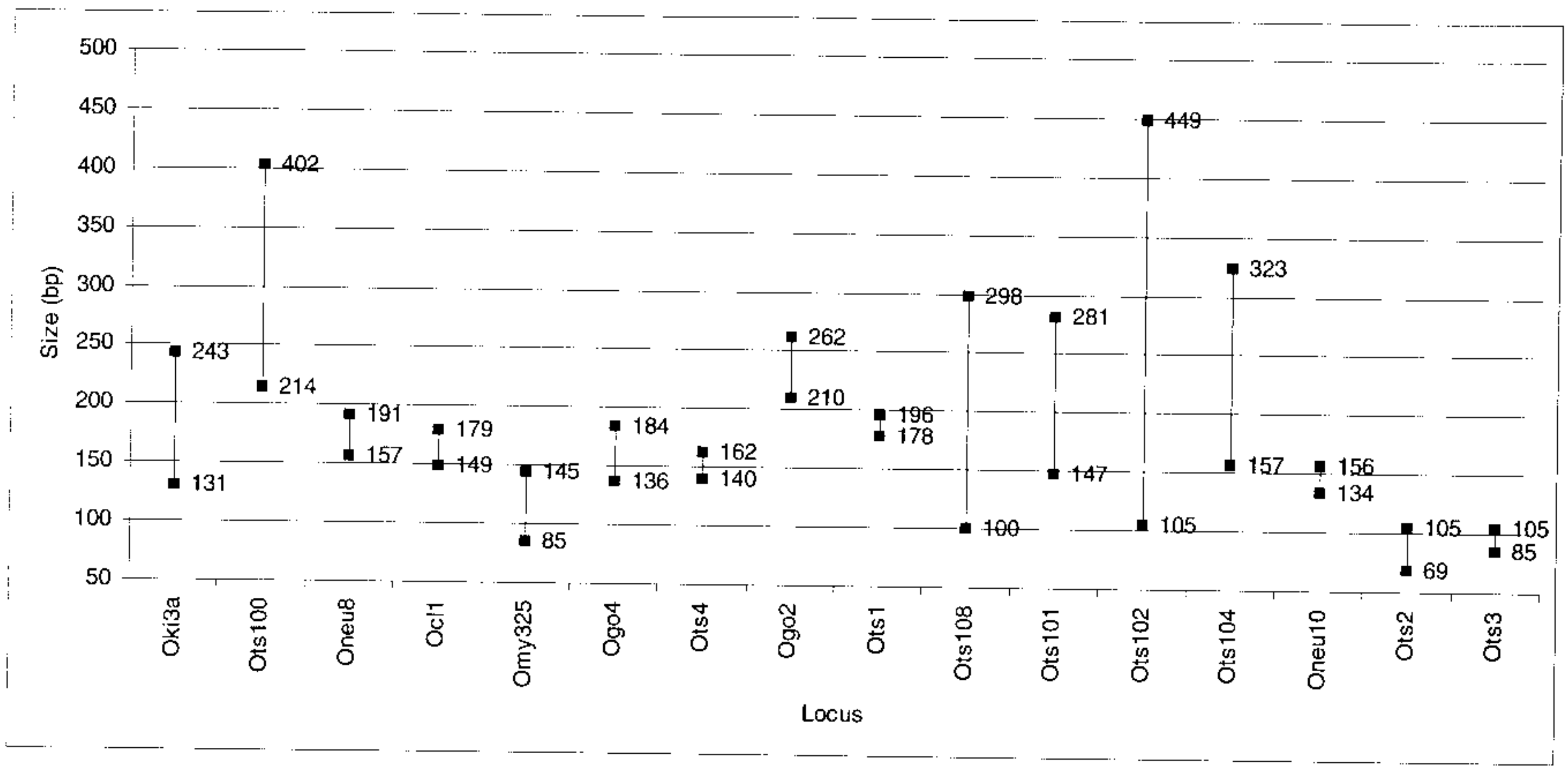

Figure 1. Estimated allelic range of 16 microsatellite loci selected for chinook salmon kinship analysis.

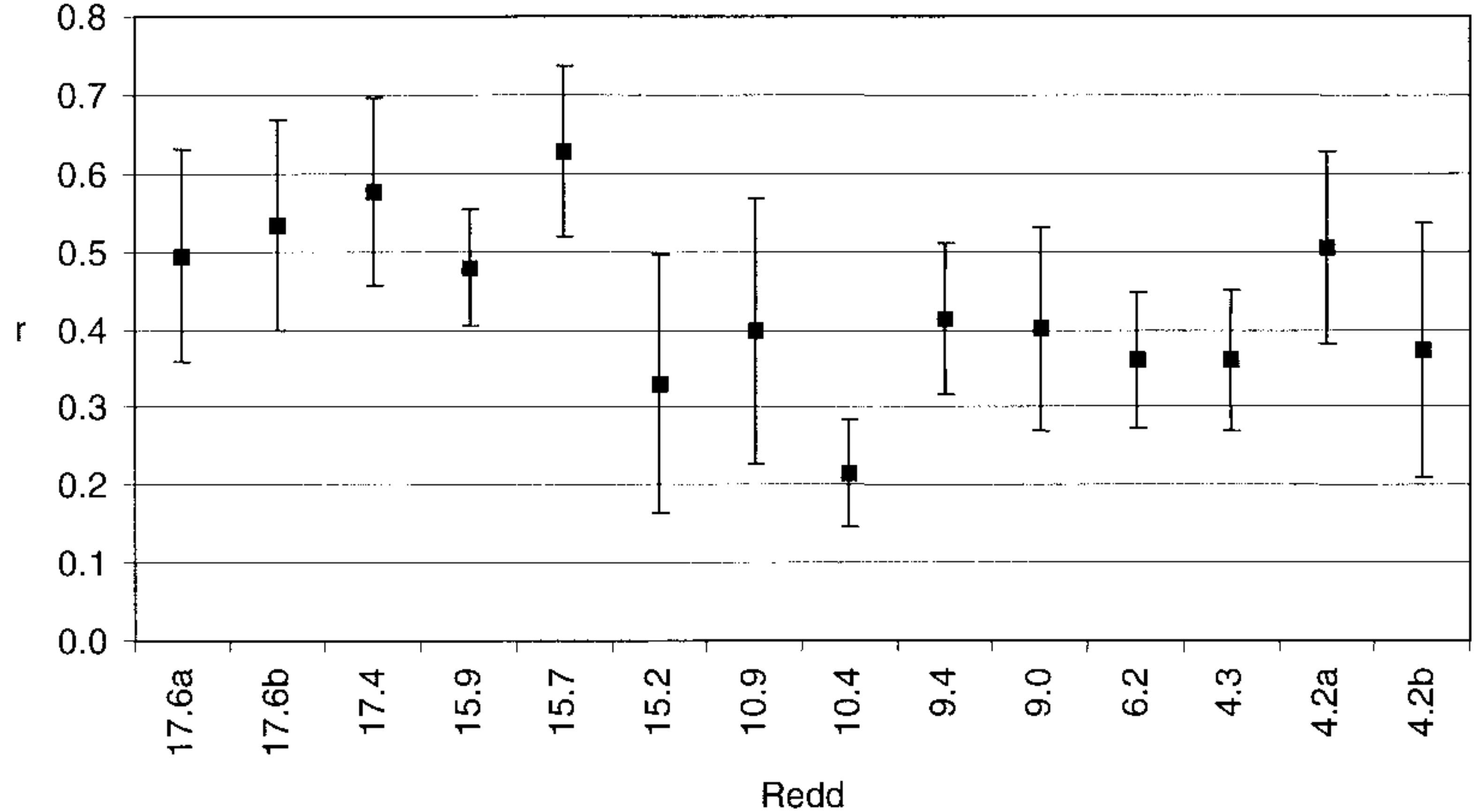

Figure 2. Estimates of relatedness among individuals within redds for F_1 Chinook salmon. The bars at each estimate indicate the 95% confidence intervals generated by jackknife sampling of 14 microsatellite loci.

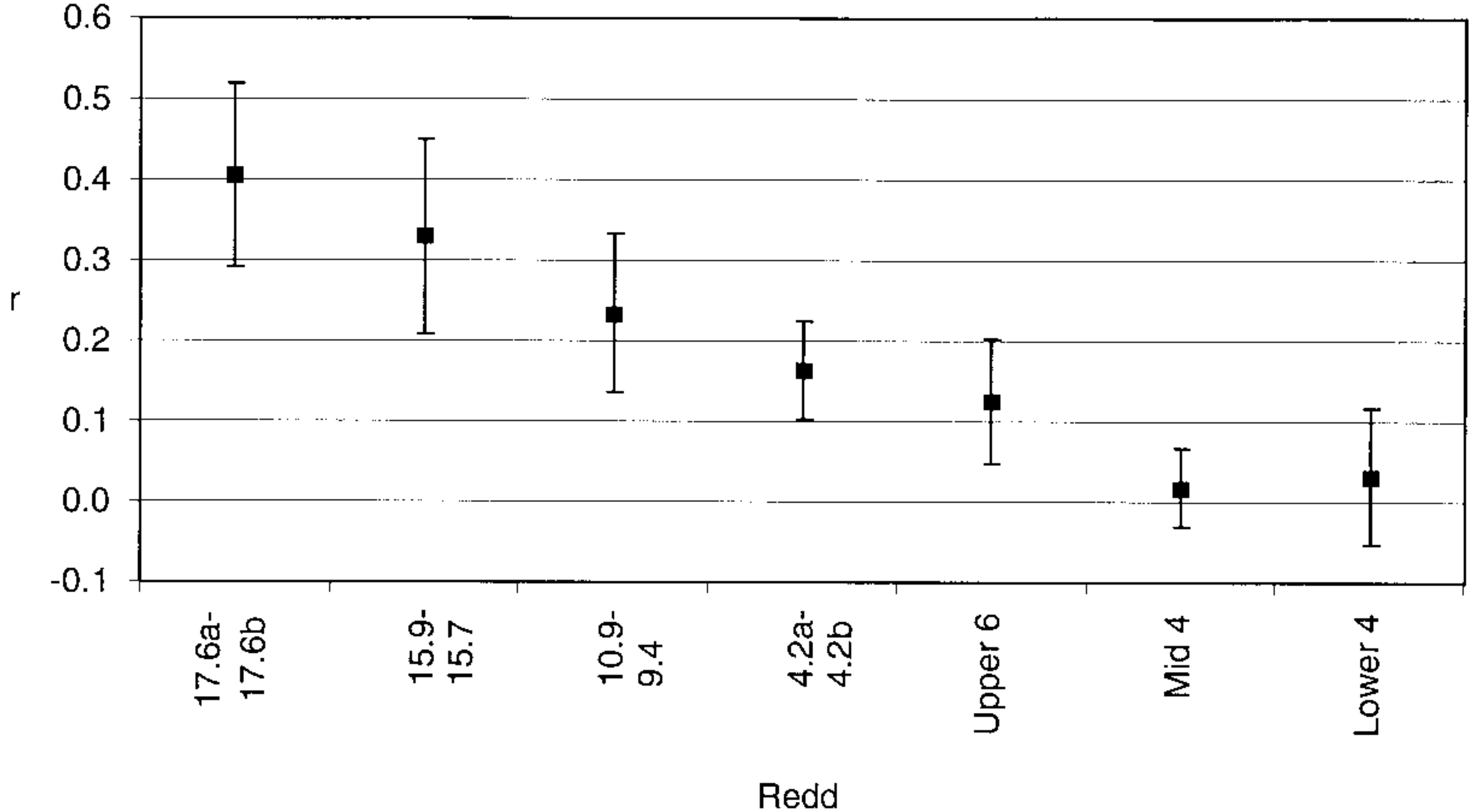

Figure 3. Estimates of relatedness among individuals from different redds for 1992 Dungeness River Chinook salmon. The bars at each estimate indicate the 95% confidence intervals generated by jackknife sampling of 15 microsatellite loci.

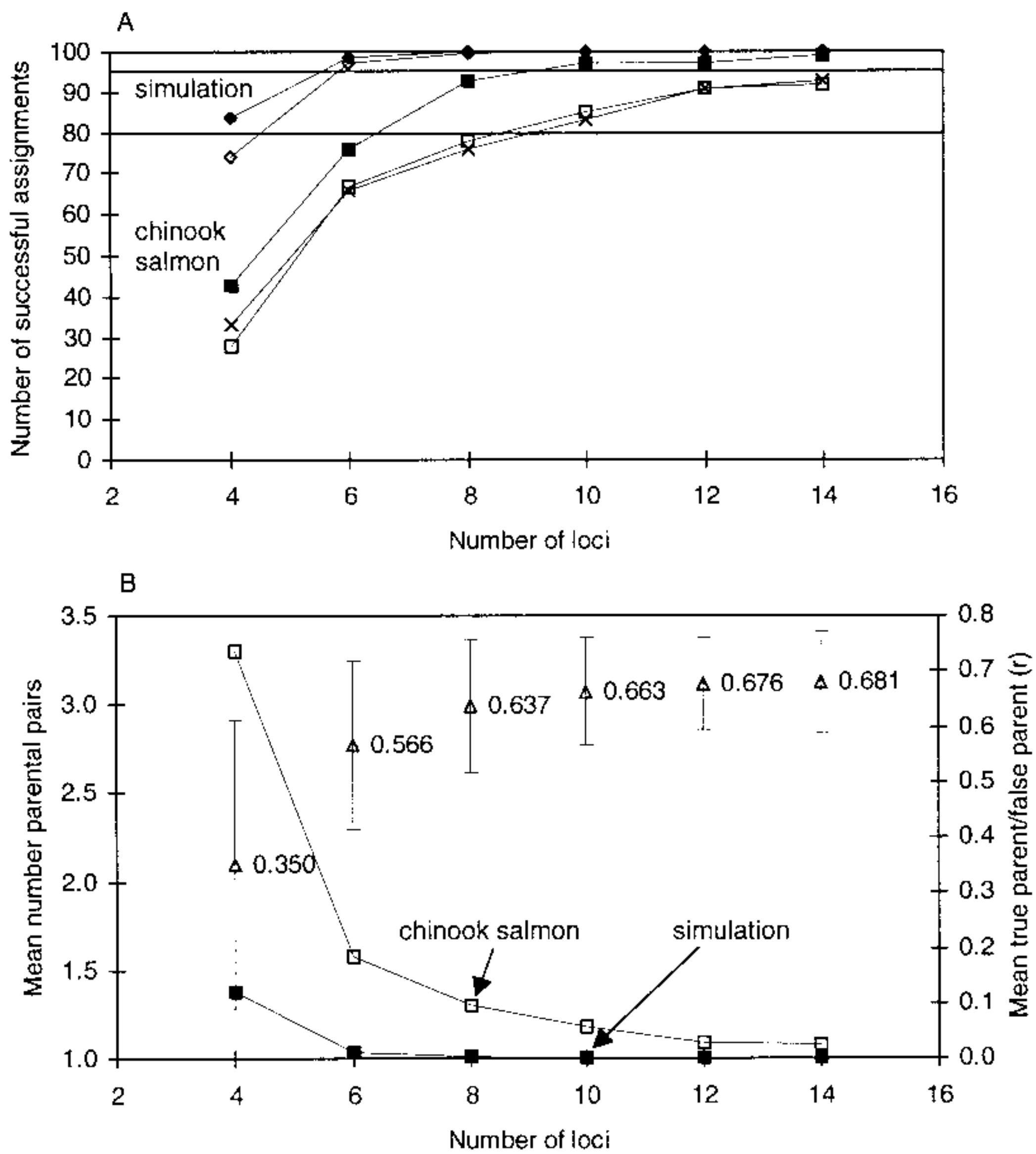

Figure 4. (A) Relationship between parentage assignment success and number of loci for the simulated pedigrees (◆, ◇) and chinook salmon pedigree (■, □, x). Parentage analysis was conducted using exclusion (◇, □) and exclusion + PPO likelihood analysis (◆, ■) for both pedigrees, and SPO likelihood analysis (x) for the chinook salmon pedigree. (B) Relationship between mean number of non-excluded candidate parent pairs per offspring and number of loci for the simulated pedigrees (■) and chinook salmon pedigree (□). Also shown are mean relatedness estimates (△) for all true parent/false parent pairs in the chinook salmon pedigree. Error bars denote standard deviation of the mean relatedness estimate.