REDUCED GENETIC DIVERSITY AND HIGH VARIANCE IN REPRODUCTIVE SUCCESS IN CAPTIVE-BRED PINTO ABALONE, Haliotis kamtschatkana

A Thesis

Presented to

The Faculty of Graduate Studies

of

The University of Guelph

by

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In partial fulfillment of requirements

for the degree of

Master of Science

April, 2007

Your file Votre référence
ISBN: 978-0-494-33882-7
Our file Notre référence
ISBN: 978-0-494-33882-7

ABSTRACT

REDUCED GENETIC DIVERSITY AND HIGH VARIANCE IN REPRODUCTIVE SUCCESS IN CAPTIVE-BRED PINTO ABALONE, Haliotis kamtschatkana

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To mitigate the decline of threatened species, captive breeding programs are often used to augment diminishing populations. Success of these programs depends on introducing captive-bred animals to their historical range without altering wild genetic diversity or patterns of local adaptation. I tested for differences in genetic diversity between three captive-spawned and one wild population of the threatened pinto abalone, *Haliotis kamtschatkana*, and found a significant reduction in allelic richness among captive-bred groups. DNA-based pedigree reconstruction was used to test the hypothesis that variance in reproductive success is in part responsible for the observed reduction in genetic diversity. I found that several abalone released gametes during spawning, but failed to produce any offspring. Even among abalone that had successful fertilization, there was high variance in the number of offspring produced. Elucidating factors responsible for altered genetic structure and reduced genetic diversity will directly aid the conservation of this threatened species.

ACKNOWLEDGEMENTS

This project could not have been completed without the help of many amazing people: Wild abalone samples were donated by R. Withler and J. Supernault from the Pacific Biological Station in Nanaimo BC, the completion of this thesis was highly dependant on their generous contribution; K. Hind and D. Steinke at the Canadian Centre for DNA Barcoding sequenced over a hundred samples of wild abalone for use in this study; A. Holliss and E. Holmes taught me how to master the ways of the Beckman CEQ 8000 (and provided much entertainment while I worked in their lab); M. Morrissey was kind enough to volunteer the use of his unpublished software program (Pedantix), which facilitated simulations of Pedigree 2.2. I would also like to thank D. Renfrew, G. White, and J. Richards at the BHCAP hatchery for allowing this project to go forward. A special thanks to J. Tosh, S. Lum, M. Lloyd, A. Grifiths, and H. Alexander for their help collecting tissue samples and tagging the abs. E. Boulding, K. Hind, H.J. Lee, M. Culling, and H. Freamo were all subject to much rougher drafts of this thesis and their input was highly appreciated. Thanks to the members of the Bogart-Boulding-Fu lab discussion group, for a substantial contribution to my knowledge of molecular ecology and population genetics. Special thanks to E. Boulding, as well as my committee members R. Danzmann and J. Bogart for help along the way.

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INTRODUCTION

Genetic Diversity

Genetic diversity is the primary biological resource within a population and represents the total adaptive potential of future generations (Allendorf and Phelps 1980). Natural selection acts on heritable genetic variation, and will increase the fitness of individuals with the most favorable variants. As a consequence, the frequency of the favored variant will increase within a population. However, fitness is ephemeral, and variants that are currently neutral or deleterious may be favored if there is a change in the environment. Similarly, variants with the highest fitness may be at a disadvantage if changes in the environment favor new traits (Halliburton 2004). As a result, the future adaptive potential of a population is dependent on there being a large pool of genetic diversity available to cope with random changes in the environment.

For selectively neutral molecular markers (i.e. loci that occur in noncoding regions of the chromosome, and are not tightly linked to coding genes), genetic structuring of alleles across populations results from an interaction between random genetic drift, mutation, and migration (Kimura 1983). Genetic drift is the stochastic change in gene frequencies due to sampling error in the production of the next generation (Wright 1950). The effect of genetic drift is much more prominent in small populations where the breeding parents may not represent the total variation available within the meta-population (Allendorf and Phelps 1980). Drift is also increased among isolated populations where there is little input of novel variation from migrants (Wright 1932; Foll and Gaggiotti 2006). An

extreme example is the colonization of a new area by a few individuals or a single mated female (Mayr 1963; Nei et al. 1975). The genetic bottleneck imposed by such a founder effect will reduce the evolutionary potential of a species by truncating the diversity that was historically found in the population. The result is that these small populations are much more sensitive to future stochastic environmental events and subsequently have a greater risk of extinction (Frankham et al. 2002).

Small isolated populations are also more likely to experience an increased level of inbreeding due to their reduced number of mating partners. Inbreeding increases the probability that an individual will receive two alleles that are identical by descent (i.e. increases the number of homozygotes). This usually results in the fixation of mildly deleterious recessive alleles that would not otherwise become expressed in an outbreeding population. Several recent studies with *Drosophila* have shown that inbreeding has a direct impact on the extinction risk of small populations (Bjilsma et al. 2000; Woodworth et al. 2002; Armbruster and Reed 2005).

It should be noted that for quantitative trait loci there is evidence that additive genetic diversity may actually increase after a genetic bottleneck (Carson 1990). Bryant et al. (1986) created short bottlenecks of different sizes on populations of *Drosophila* and found that heritability and genetic variation increased for eight different morphometric traits. It has been suggested that this may result from the disruption of co-adapted complexes of genes that are usually balanced by the high fitness of complex heterozygotes (Carson 1990). In this

scenario, epistatic variance is converted to additive genetic variation, which is then subject to natural selection. However, Van Buskirk and Willi (2006) question whether this sort of increase in additive genetic variation will impact the viability of the bottlenecked population. To resolve this issue, Gilligan et al. (2005) suggest that experimental data from a broader range of species is needed with a focus on variation associated specifically with characters that strongly influence fitness.

Captive Breeding

Environmental destruction caused by anthropogenic factors such as habitat loss, pollution, and the introduction of invasive species has put many species at risk of extinction (Soule and Mills 1998; Frankham 2003; Wang et al. 2005). To aid the conservation of threatened or endangered species, captive breeding programs are often used to assist species that will not return to historical levels of abundance without human intervention (see Fiumera et al. 2000; Norton and Ashley 2004; Russello and Amato 2004, Theodorou and Couvet 2004). The goal of these programs is to re-introduce captive-bred animals to their historical range or to supplement areas where there are still a few remaining conspecifics (Nicoll et al. 2004). Conceptually, the formation of a new captive-breeding population is analogous to a natural founder event where a small number of individuals or even a single mated female colonize a new area. As a result, the success of a captive breeding program depends on our ability to minimize the genetic deterioration associated with small population size.

Populations that are maintained over several generations may experience natural selection for traits that increase their adaptation to captivity (Price 1970; Woodworth et al. 2002). Experiments have shown that adaptation to captivity can drastically reduce the fitness of captive-bred species upon reintroduction to the wild (McPhee 2003). In addition, selective pressures in captivity tend to be significantly altered from those in the wild and may facilitate the accumulation of deleterious genes that would otherwise be kept at low frequency (Theodorou and Couvet 2004). The result is that without proper management, supplementation programs using captive-bred animals may actually increase the genetic load on remaining wild populations (Theodorou and Couvet 2004).

To prevent inbreeding and slow genetic deterioration among captive populations, it is useful for conservation biologists to have information about the relatedness of the founding animals prior to captive breeding (Selvamani et al. 2001; Jones et al. 2002). Blouin (2003) reviews several algorithms and software programs that use data from neutral genetic markers to reconstruct pedigree in wild populations. These reconstructed pedigrees can then be used to prevent the mating of closely related individuals, and to estimate variance in reproductive success of the breeding population. This is especially useful when studying animals where the pedigree cannot easily be tracked and controlled. For example, observation-based pedigree assignment is very difficult among organisms that lack parental care, have polygamous mating systems, or that reproduce by broadcast spawning (Boudry et al. 2002; Wilson and Ferguson 2002; Herbinger et al. 2006).

The recent proliferation of microsatellite DNA markers provides a useful tool for measuring levels of genetic diversity and determining kinship in captive populations (Queller et al. 1993). Microsatellite loci are non-coding segments of DNA with very short nucleotide motifs that are repeated in tandem. Each locus has a large number of co-dominant alleles consisting of different numbers of repeat units. For example, a particular microsatellite locus may consist of repetitions of the base sequence 'CA', and different alleles will be characterized by the number of times that this base sequence is repeated. Alleles can then be scored based on their length (Queller et al. 1993). Given that microsatellites are highly polymorphic, co-dominant, and are very easy to score, they are ideal tools for studies of kinship and for monitoring genetic diversity among wild and captive populations.

Pinto Abalone

Pinto (or northern) abalone, *Haliotis kamtschatkana*, are subtidal marine mollusks that occur along the northeastern coast of the Pacific from Baja California to Alaska (Campbell 2000). They have a patchy distribution throughout their range and inhabit exposed and semi-exposed rocky shores. Pinto abalone reach reproductive maturity at approximately three years of age (shell length between 50-65mm), and reproduce by broadcast spawning. Fertilization is followed by a short (five-six day) free-swimming larval period that facilitates dispersal (Sloan and Breen 1988). Studies of pinto abalone in captivity suggest that they may grow by only 10mm per year (J. Tosh, unpublished data).

Historically, *H. kamtschatkana* was a commercially important marine shellfish. However, over-fishing resulted in a 75-80% decline in this species abundance during the 1980's and by 1990 the pinto abalone fishery in British Columbia was closed (Sloan and Breen 1988). Recent studies suggest that wild abalone stocks are currently not returning to historical levels despite the complete ban on harvesting (Campbell 2000). It has been suggested that high levels of poaching, low reproductive success, and possibly the reintroduction of sea otters to their native habitat, are responsible for continued decline of *H. kamtschatkana* (Watson 2000; Withler et al. 2003). *H. kamtschatkana* is currently listed as a threatened species by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) and under Schedule 1 of the Species at Risk Act (SARA).

To mitigate the continued decline of *H. kamtschatkana*, the Bamfield Huu-ay-aht Community Abalone Project (BHCAP) was created with a primary goal of conserving the pinto abalone. BHCAP runs a captive breeding program for pinto abalone in Bamfield, British Columbia. They have been facilitating controlled abalone spawning since 2001 and using the resulting captive-bred offspring to augment the threatened wild population.

Broadcast-spawning marine organisms present unique problems for captive breeding programs. During broadcast spawning, all individuals in an aggregation eject their gametes into the water column, and fertilization is dependant on the coordinated timing of a large number of individuals in close proximity to each other (Hedgecock 1994). The resulting offspring may form a

highly complex pedigree involving all-possible crosses between a large number of males and females (Levitan 2005). Conversely, broadcast spawning has the potential to produce a very large population from a limited number of parents (Smith and Conroy 1992; Boudry et al. 2002). This is problematic from a conservation standpoint given that census population size may be extremely large despite there being a small effective population size. Hedgecock (1994) describes this phenomenon as the 'sweepstakes hypothesis' in which high fecundity and high early mortality among marine spawning organisms may lead to a substantial variance in reproductive success. The result is that a small number of breeders can replace the entire population in each generation, causing substantial reduction in effective population size and imposing genetic structure between generations.

My first research question asks whether captive-breeding has had a detectable impact on the genetic diversity of pinto abalone offspring produced at the BHCAP facility. I hypothesize that captive breeding has imposed a genetic bottleneck on each of the first generation strains of captive-bred offspring. If this is true then I expect to find a significant reduction in microsatellite allelic richness and differences in genetic structure between wild and captive-spawned groups of pinto abalone.

My second question asks why genetic diversity and structure are being altered. I will test the hypothesis that reduced genetic diversity and altered genetic structure is in part the result of high variance in reproductive success among the broodstock. If this hypothesis is correct, then pedigree reconstruction

will reveal a deficiency in the observed number of full-sib families. I also predict that among the parents who contributed genes, there will be a high variance in the number of offspring produced by each unique male-female pairing.

Elucidating factors responsible for altered genetic structure and reduced genetic diversity will directly aid in the conservation efforts for this threatened species. In addition, these results will be valuable to other captive breeding programs, especially those that deal with broadcast spawning species in which seemingly large populations are in fact derived from a very small number of parents.

METHODS

Sample Collection and Analysis

All captive-bred pinto abalone offspring examined in this study were the product of pre-existing spawning events that had taken place before the inception of my research project. As such, I had no input into the spawning protocol and was dependant on hatchery records to determine how many wild males and females were used to create each group of captive-bred offspring. The number of wild abalone (broodstock) that BHCAP hatchery managers used to spawn each captive-bred group is consistent with protocols used to propagate abalone for aquaculture in other hatcheries (Smith and Conroy 1992).

Before each captive spawning at the BHCAP facility, wild broodstock were visually inspected to assess the ripeness of the gonads. Six to ten mature abalone were then chemically induced to spawn in separate individual tanks; this is advantageous as it assures that gametes are contributed from all broodstock.

Gametes from each individual were then collected and diluted before being mixed together; this helps to equalize the genetic contribution from each parent and prevents polyspermy that results when sperm concentrations are too high (D. Renfrew, pers. com.). After fertilization, the resulting nonfeeding larvae are kept in tanks at a land-based holding facility where they settle as post-larval juveniles (D. Renfrew, pers. com.).

Between May 2005 and August 2006 I sampled offspring that were the product of three captive spawning events. These three captive-bred groups were spawned in 2002 and 2003 and will be referred to throughout this thesis as the B-group, the R-group, and the T-group (Table 1). All of the broodstock used in these captive-spawnings were from the wild, which made these offspring the first generation of captive-bred abalone at the BHCAP facility. In most cases, the parents of each spawning group had already been returned to the wild; however I collected tissue from any broodstock that were still available for sampling.

In addition to the three captive-bred groups, I also obtained DNA from a large number of wild abalone. All wild *H. kamtschatkana* DNA used in this study was donated by the Pacific Biological Station in Nanaimo BC. These wild DNA samples were from abalone collected in Trevor Channel near Bamfield British Columbia in 2001 (for complete sampling and DNA extraction protocol see Wither et al. 2003), and are representative of the wild Barkley Sound metapopulation that is being supplemented by captive-breeding.

DNA Extraction, Microsatellites, and Genotyping

From each abalone a single epipodal tentacle was removed using forceps and stored in 95% ethanol at –20 °C prior to DNA extraction. Removal of a single epipodal tentacle is considered non-destructive, and does not impact the survival of the abalone (Withler et al. 2003). DNA was extracted from tissue samples using a Qiagen DNEasy 96-well extraction kit™ according to the supplier's protocol.

I screened seven microsatellite DNA loci, Hka12, Hka37, Hka40, Hka43, Hka48, Hka56, and Hka65, which were developed by Miller et al. (2001), GenBank accession AY013572, AY013575, AY013576, AY013577, AY013578, AY013579, AY013581 for use in this study (table 2). I discarded Hka12 and Hka48 because they had a high polymerase chain reaction (PCR) failure rate and were very difficult to score with accuracy.

PCR was carried out using a RoboCycler® 96 Gradient Cycler with Hot Top PCR machine (Stratagene, Canada); all PCR reagents were purchased from Invitrogen unless otherwise indicated. PCR amplification was carried out in a 10µl reaction mixture that included 1.0µl of undiluted DNA template, 80µM of dNTPs, 0.25 units of Taq DNA polymerase, either 2.7mM $MgCl_2$ (Hka40 and Hka43) or 0.3mM $MgCl_2$ (Hka37, Hka56, and Hka65), 1.25µM of each primer, 1µl off buffer, and ultraPURE™ distilled water to volume. The forward primer of each pair was ordered from Proligo (Sigma-Aldrich, USA) and was labeled at the 5' end with either Beckman fluorescent dye D3 (Hka65) or Beckman fluorescent dye D4 (Hka37, Hka40, Hka43, and Hka56). PCR consisted of an initial

denaturation step of 94°C for 5 min followed by denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec (Hka37, Hka56, and Hka65), 48°C for 30 sec (Hka43), or 47°C for 30 sec (Hka40), followed by extension at 70° for 45 sec (Hka37, Hka40, Hka43, Hka65) or 30 sec (Hka56). Thirty-five cycles were used for Hka37, Hka40, and Hka56, 32 cycles were used for Hka65, and 38 cycles were used for Hka43.

PCR product was run on a 2% agarose gel and viewed using an AlphaImager™ 3400 Gel Doc System (Alpha Innotech, USA) to determine whether the reaction was successful. To score the allele sizes, successfully amplified PCR product was then run on a Beckman Coulter CEQ 8000 Genetic Analysis System using the manufacturer's recommended protocol except that only half as much 400bp Beckman Coulter size standard was used. Each plate was run with a sample of known genotype to correct for possible scoring differences between runs. When samples were re-run on the Beckman machine, the size of the alleles varied between runs by fewer than 0.5 nucleotides.

Genetic Diversity Analyses

(i) Variation within populations:

Population-genetic statistical analyses were carried out on the wild and hatchery samples to test the hypothesis that genetic diversity among captive-bred groups was significantly reduced relative to the wild samples. Arlequin version 3.1 (Excoffier et al. 2005) was used to estimate the total number of alleles per locus as well as the expected and observed heterozygosity. In addition, allelic richness for each locus in each group was estimated using

FSTAT version 2.9.3 (Goudet et al. 2001). Allelic richness (R_S) is a rarefied measure of the abundance of alleles that is independent of sample size; in our study the number of alleles was standardized to 60 diploid individuals per group. A nonparametric analysis of variance (Mann-Whitney U test) was then used to test for differences in mean allelic richness, and for differences in expected heterozygosity between wild and hatchery samples using SPSS version 12. For this test, each locus from each sample group was used as a separate independent replicate.

For all loci, GenePop version 3.4 (a web-based update of version 1.2, Raymond and Rousset 1995a, available at: http://wbiomed.curtin.edu.au/genepop/) was used to determine whether the inbreeding coefficient, F_{IS}, was significantly different from zero. I also carried out a pairwise test for linkage disequilibrium for each locus in each sample using GenePop 3.4. For each of these tests, p-values were sequentially Bonferroni corrected for multiple tests (Rice 1989).

(ii) Population differentiation:

From a conservation standpoint, the ideal situation would be no population structure between wild and hatchery groups (i.e. captive-bred animals would have the same allele frequency distribution as the wild population). The following analyses were carried out using Arlequin 3.1 to test the hypothesis that captive-bred individuals show significant differences in genetic structure compared with the wild population. I also tested whether there was significant population

differentiation among each of the three captive-bred groups despite the fact that they were produced from the same gene pool.

Genetic distance between all pairs of samples was estimated using a pair wise F_{ST} (Weir and Cockerham's Θ; Weir and Cockerham 1984). I used 1000 permutations and a significance level of 0.05. In addition, an exact test of population differentiation was used to test the null hypothesis that there was a random assortment of alleles between all pairs of populations (Raymond and Rousset 1995b). I used 10^5 steps in the Markov chain with a burn in of 10000 steps and evaluated the significance after a Bonferroni correction for multiple tests (Rice 1989).

Pedigree Analysis

Pedigree analyses were used to test the hypothesis that high variance in reproductive success of the broodstock contributes to reduced genetic variation in captive-bred abalone. I used a pedigree reconstruction algorithm to identify full-siblings (i.e. families of offspring that share the same two parents) for each spawning group. The resulting full-sib partitions were then used to determine (a) whether all possible crosses occurred between parents present during each captive spawning, and (b) whether certain broodstock produced more offspring than others.

Given that abalone reproduce by broadcast spawning, all possible crosses of males and females could potentially occur during a group-spawning event (Levitan 2005). This fully-factorial mating system greatly increases the complexity of the pedigree and may reduce the ability to accurately partition

individuals into correct full-sib families (Herbinger 2005). Further difficulty in elucidating full-sib groups will occur if some families are proportionately smaller than the population average (Herbinger 2005).

To reconstruct pedigree within each hatchery group, I used Pedigree version 2.2 (Smith et al. 2001; Herbinger 2005), which uses a Markov chain Monte Carlo (MCMC) algorithm to reconstruct the pedigree of individuals using co-dominant marker data in the absence of parental genotypes. This is a user-friendly web-based program for pedigree reconstruction with only three parameters that must be optimized by the user. These are: number of iterations, temperature (i.e. how likely the algorithm is to remain on a local maximum on the likelihood surface), and weight (increased weight increases the coalescence of full-sib families in situations where there are some families are much larger than others, or when the offspring are comprised of a mixture of full and half sibs) (Herbinger 2005).

Before reconstructing the pedigree of captive-bred offspring, I ran several simulations to assess the efficiency of Pedigree 2.2 at elucidating complex factorial pedigrees. All of these simulations were run using offspring genotypes created by the program Pedantix (unpublished computer software package, M.Morrissey, University of Guelph, Ontario), which produces simulated multi-locus data sets using the actual microsatellite gene frequencies of the real population. The user inputs the number of breeding males and females, the number of offspring to be produced by each male/female cross, as well as the percentage of missing data and the error rate of allele scoring. Pedantix then

assigns genotypes to each of the simulated parents based on the frequency of alleles in the true population. Simulated offspring genotypes are then created by assuming Mendelian inheritance of alleles from the simulated parents using the user-specified male-female crosses.

For each captive-spawning group, actual allele frequencies and the expected number of male and female parents were used to create simulated data sets. These were used to test the ability of Pedigree 2.2 to accurately partition offspring into the correct full sib group. I created simulated data sets with different levels of factorial crosses, and with high variance in family sizes to rigorously test Pedigree 2.2.

After resolving the optimal parameters for each group (Table 3), separate runs were completed with an entire locus omitted from the data each time. Full-sib partitions were compared between these runs to a) confirm the likelihood that the given partition was accurate, and b) to test whether certain loci may have a strong impact on the results. Removing a single locus from the pedigree analysis had almost no effect on the partitioning of full-sib individuals. This suggests that there were an ample number of loci and that none of them significantly affected the results (i.e. each combination of four loci gives same result as all five loci).

Pedigree 2.2 was also used to reconstruct the genotype of both parents of each full-sib family. I compared reconstructed genotypes of putative parents to broodstock for which DNA samples were still available. This allowed me to determine which sampled parents successfully produced offspring.

In addition, three large reconstructed full-sib families were used to test for segregation distortion and deviation from expected Mendelian inheritance of alleles among the loci used in this study. This analysis was carried out using LINKMFEX (Danzmann 2005), which uses multi-locus genotypes of two parents and their full-sib offspring to estimate recombination rates, deviation from Mendelian expectations, and to elucidate the presence of linkage groups.

mtDNA Sequencing and Analysis

I further tested the accuracy of the pedigree reconstruction by verifying that abalone within each full-sib group share the same haplotype at the mitochondrial locus, cytochrome c oxidase subunit 1(CO1). Given that mitochondrial DNA (mtDNA) is maternally inherited in the genus *Haliotis* (Metz et al. 1998; Gruenthal and Burton 2005), and that the mutation rate is less than 9.7 x 10^{-8} per site per generation (Denver et al. 2000), every offspring that Pedigree 2.2 correctly places within a full-sib family should have the same CO1 haplotype.

A preliminary study using DNA template from the wild population was carried out to assess the natural abundance of mitochondrial CO1 diversity. I initially sequenced a 452bp section of the CO1 gene from 18 wild individuals; an additional 126 sequences were later obtained from the Canadian Centre for DNA Barcoding, these sequences were from the same DNA template used in the microsatellite analysis of the wild population, and each PCR was carried out using the same primers and protocol described below. To my knowledge, this is the first study to examine CO1 haplotype diversity among wild pinto abalone in Canada. After confirming the presence of variation at the CO1 locus among the

wild abalone (Table 4), several individuals from each reconstructed family were sequenced to verify the accuracy of the pedigree reconstruction.

For each individual, DNA was amplified using abalone-specific mtCO1 primers developed by Metz et al. (1998) (GenBank accession AF060845. Forward primer: 5'-TGATCCGGCTTAGTCGGAACTGC-3'; reverse primer: 5'-GATGTGTTGGAAATTACGGTCGGT-3'). Site one in our alignment is equal to nucleotide position 90 in the full AF060845 sequence. All reagents were purchased from Invitrogen unless otherwise indicated. Each 50μl PCR contained 2.5μl of each primer, 0.25 units of Taq polymerase, 1.5mM of $MgCl_2$, 0.08 mM of dNTPs, 2μl of undiluted DNA template, 5μl reaction buffer, and ultraPURE™ distilled water to volume. PCR was carried out using a RoboCycler® 96 Gradient Cycler with Hot Top PCR machine. Each PCR consisted of an initial denaturation step of 5 minutes at 95°C followed by 32 cycles of 95°C for 30 seconds, 52°C for 40 seconds, and 72°C for 45 seconds. There was a final extension step of 5 minutes at 72°C. PCR products were run on a 2% agarose gel and viewed using an AlphaImager™ 3400 Gel Doc System (Alpha Innotech, USA) to determine whether the reaction was successful. mtDNA was then purified from solution using a GFX PCR DNA and Gel Band Purification Kit™ (Amersham Biosciences) according to the manufacturers' protocol. Purified DNA was subjected to direct sequencing using diluted (5 pmol) forward primer and BigDye Terminator 3.1 Cycle Sequencing Ready Reaction Kit™. DNA sequencing was carried out on an ABI 3730 DNA Analyzer (Applied Biosystems) and analyzed with ABI prism DNA sequencing Analysis Software version 3.4.

DNA sequences were edited using Chromas version 1.45 (Conor McCarty, Griffith University, Australia; available at: http://www.technelysium.com.au/chromas.html). DNA alignment was carried out using Clustal W version 1.83 (Chenna et al. 2003). The haplotypes of offspring in each reconstructed full sib family were compared to ensure that each individual within a reconstructed family shared the same haplotype and to determine the error-rate of each of the pedigree reconstructions. A rarefied measure of haplotype diversity was also estimated for the wild and captive bred abalone using the program Rarefac (Petit et al. 1998).

RESULTS

Genetic Diversity

All five microsatellite loci were polymorphic in each of the three spawning groups (Appendix A). Within the three captive-bred groups, the number of alleles per locus (N_A) ranged from 5 to 15, whereas within the wild samples there were between 12 and 28 alleles per loci. The Mann-Whitney U test revealed that captive-bred samples had a significantly lower allelic richness (mean R_S=8.5) than the wild samples (mean R_S=18.8) (p=0.001). Captive-bred abalone also demonstrated a small but significant reduction in expected heterozygosity compared to the wild group (mean wild H_E = 0.88, mean captive-bred H_E = 0.72; Mann-Whitney U test, p=0.016), however, there was no significant difference in expected heterozygosity between different captive-bred groups (p> 0.1).

Captive-bred samples also showed significant deviations from Hardy-Weinberg equilibrium (HWE) (exact test, P<0.001) and significant heterozygote

excess (F_{IS} values that were significantly less than zero) (Table 5). In contrast, the wild samples were all in HWE.

The wild population did not show any pairwise departures from linkage equilibrium. This was consistent with the extensive wild study carried out by Withler et al. (2003). However, among the hatchery samples there were several deviations from linkage equilibrium (Appendix B).

Pairwise F_{ST} values were highly significant between all four sampled groups ($p<0.0001$) (Table 6). In general, pairwise F_{ST} values obtained in this study indicate a low level of population differentiation for all pairwise comparisons ($F_{ST} < 0.15$, Frankham et al. 2002). However there was a slight trend towards higher population differentiation between captive-bred groups than for each captive-wild comparison. Similarly, the exact test for population differentiation indicated significant differences between captive-bred groups, but was not significant in any of the captive-wild pairwise comparisons (Table 7).

Pedigree Analysis

Simulated data confirmed that Pedigree 2.2 was very accurate at grouping related individuals into full-sib groups; however it sometimes failed to combine all members of a large full-sib family into a single group, even when the parameter that increases the coalescence of these groups was set quite high (i.e. weight of 10 or higher). Instead, the program would place some members of a large family into smaller full-sib groups (≤4 individuals). Individuals placed in one of these small families were true full siblings; however they should have been placed with the rest of their full-sib group and did not actually represent a unique family

(Butler et al. 2004). For this reason, I chose to only accept a true family as one that has five or more individuals; full-sib partitions having less than five individuals were removed from the pedigree. From the perspective of reconstructing pedigree to minimize inbreeding, accepting the small families as unique groups would run the risk of mating them with other members of their true family. Conversely, by removing these partitions I do run the risk of missing very small full-sib families and thus of underestimating the total number of families.

Within each of the three spawning groups there was a high variance in full-sib family size among the sampled individuals. Family size ranged from five to 108 offspring (Appendix D), and each group is characterized by one proportionately large family and several much smaller families (Figure 1). Also, for each spawning group there were fewer observed families than the maximum number possible if all male-female crosses had occurred.

Using the parental genotype reconstruction function in Pedigree 2.2 combined with mitochondrial haplotype information for each full sib group (see below), and the genotypes of broodstock that were sampled, I was able to determine which broodstock were the parents of each family (Table 8, Figure 2a-c). I found that each of the three captive-spawning events had a single male or female parent that dominated. This was most exaggerated in the R-group where a single male was responsible for all of the offspring. In addition, reconstructing parental genotypes revealed the presence of parents in the R-group that should not have been involved in that spawning. This indicates that there may also be

errors in the spawning records at the captive-breeding facility (D. Renfrew, pers. com.).

Analysis of three large reconstructed families revealed evidence for segregation distortion among two of the loci used in this study. In general, recombination rates between all pairwise locus comparisons were high (Appendix E); however two loci (Hka37 and Hka 65) displayed significant deviations from Mendelian expectations in two full-sib groups (Appendix F).

mtDNA

A 452bp segment of the CO1 gene was successfully sequenced for 71 captive-bred offspring. While there were 13 haplotypes present among the 144 wild abalone samples, only the three most common haplotypes were detected among the captive-bred abalone (rarefied haplotype richness standardized to 20 individuals: wild =5.3, captive bred =2.0).

Mitochondrial DNA sequences from the offspring were then used to corroborate the pedigree analysis. The number of sampled offspring that had been misplaced by Pedigree 2.2 ranged from one to three among captive-bred groups (b-group error = 1/20; r-group error = 2/24; t-group error = 3/27) (Appendix G). This represents a 5-11% error rate among sampled individuals.

DISCUSSION

Genetic Diversity and Structure

The results of this study reveal significant differences in genetic diversity between wild and captive-bred stocks of pinto abalone at the BHCAP facility. All three captive-bred groups are characterized by a significant reduction in

microsatellite allele richness compared to the wild group, and a slight but significant reduction in expected heterozygosity. Despite this reduction in expected heterozygosity, values of H_E are still relatively high among the captive-bred abalone. In addition, all F_{IS} values among the captive-bred offspring are negative, which indicates that there is an excess of heterozygotes relative to HWE. Heterozygote excess is consistent with having experienced a recent genetic bottleneck in which the founders originated from a large out-breeding population; Given that the wild parents were highly heterozygous and had a substantial diversity of alleles per locus, there is a high probability that the offspring received two different alleles at each locus despite the small number of breeding parents. Similar results were obtained by Herbinger et al. (2006) who found greater than expected heterozygosity among captive-bred salmon that were spawned from a small number of wild parents.

This has implications for conservation given that a common management goal among captive breeding programs is to maintain 90% of the original genetic diversity (usually expected heterozygosity) over 100 years (Frankham et al. 2002). I found that among captive-bred groups, allelic richness has been reduced by an average of 57% compared to the wild, however expected heterozygosity has only been reduced by 17%. These results demonstrate that expected heterozygosity may not be adequately sensitive to detect the effect of a recent genetic bottleneck (Nei et al. 1975; Hedgecock and Sly 1990). It is therefore essential that managers of a new captive breeding program use

multiple indices of genetic diversity to assess the genetic condition of the offspring.

Deviation from linkage equilibrium may be useful for indicating the presence of a recent population bottleneck (Bartley et al. 1992; Hansson and Westerberg 2002; Oosterhout et al. 2004). This study found no deviations from linkage equilibrium among the wild population, however there were several significant pairwise linkage disequilibrium values among the captive bred groups. This suggests that observed deviations from linkage equilibrium result from the bottleneck imposed on captive-bred offspring and not close physical linkage of alleles. Interestingly, the deviation from linkage equilibrium was most profound in the R-group in which a single male was responsible for all of the offspring. In this group alleles do not distribute randomly between loci because there is only one male contributing genes to all the offspring.

Results from the pairwise F_{ST} analysis suggest that there is slightly higher differentiation between captive-bred groups than between each captive-bred group and the wild. This trend is corroborated by the results from the exact test for genetic differentiation, which found significant differences in gene frequencies between each captive-bred group but no differentiation for each captive-wild comparison. Conceptually, the formation of each captive-bred group is similar to a source-sink (or mainland-island) model of genetic structure, where the wild (source population) provides all of the genetic diversity to each separate captive-bred group (sink populations), with no migration between different sinks (Frankham et al. 2002). Over several generations, it is likely that genetic drift will

cause small differences in genetic structure between captive groups to increase over time.

Interestingly, genetic structuring between captive-bred subpopulations may be an effective method for preserving genetic diversity (Fiumera et al. 2000). This is based on the idea that genetic drift will reduce the variability within a population but increase the variability between populations (Wright 1950; Halliburton 2004; Saillant et al. 2005); in one population a single allele will go to fixation by genetic drift, however in several small populations it is likely that different alleles will become fixed and thus some variation will be conserved (Fiumera et al. 2000). Empirical studies have shown that the maintenance of several smaller fragmented captive populations followed by pooling will retain greater genetic diversity and experience less adaptation to captivity than a single large population of equivalent total size (Margan et al. 1998).

Pedigree Analysis

In the absence of pedigree information, conservation biologists often assume that the wild founders of captive-breeding programs are unrelated (Russello and Amato 2004). However, natural populations targeted for captive-breeding have usually undergone severe demographic decline, which potentially invalidates this assumption (Herbinger et al. 2006). I used Pedigree 2.2 to test the assumption that the wild samples of *H.kamtschatkana* were entirely unrelated. This analysis revealed very little evidence for relatedness among the wild broodstock, and is consistent with a large effective population size among natural populations of *H. kamtschatkana* (Withler et al. 2003).

To ensure that all broodstock used in captive spawning actually release and contribute gametes, individual broodstock were first induced to spawn in separate tanks and their gametes were then collected and mixed together. Despite this attempt to incorporate the maximum level of available genetic diversity, reconstructed pedigree reveals that only four or five full-sib families characterize each group of captive-bred pinto abalone. This represents far fewer observed families than expected if all possible male-female crosses had occurred, and suggests that there were several broodstock who released gametes but failed to produce any offspring. Also, among the broodstock who successfully produced offspring, there is a substantial variation in the size of each full-sib family; family sizes varied from five to 108 offspring. The magnitude of variation in family sizes suggests that factors other than gamete abundance have an overwhelming impact on fertilization success. This is supported by other studies who found that sperm motility, age of the gametes, and sperm competition play much a bigger role in fertilization success than sheer quantity of gametes (Levitan 1991, Babcock and Keesling 1999; Boudry et al. 2002).

These pedigree results give evidence to support the sweepstakes hypothesis, which predicts that temporal changes in gene frequencies of marine organisms may result from substantial variance in reproductive success (Hedgecock 1994). In the wild, a small number of successful breeders can replace the entire population in each generation, causing a substantial reduction in effective population size and significant genetic structuring between generations (Li and Hedgecock 1998). This was observed in each captive-bred

group of *H. kamtshatkana* where high variation in reproductive success caused genes from large families to dominate the genetic structure of each spawning group. As a result the effective population size is small despite there being thousands of offspring produced in each group (Boudry et al. 2002).

The results from the mitochondrial DNA analysis demonstrate a variable error rate among groups, with 5-11% of individuals being placed within the wrong maternal group. This error rate reflects the difficulty of reconstructing pedigree among offspring produced from a factorial mating system, even when highly polymorphic markers are used. Such systems are composed of many full-sib groups nested within half-sib families, which greatly reduces the resolution power of the algorithm used by Pedigree 2.2 (Herbinger 2005).

Butler et al. (2004) tested four different pedigree reconstruction algorithms to evaluate the impact of common sources of error on the accuracy of the full-sib partitions. The four sources of error they examined were: mutation or human error (where an allele in the data set is randomly changed to another allele present at the same locus); size off by one allele (where an allele at a single locus is miss-scored by one repeat unit either up or down); the presence of null alleles (where heterozygotes are incorrectly assigned as homozygotes); and genotype inversion (where the genotype of two individuals are accidentally switched at one locus). Interestingly, they found that all of the algorithms, including the one used in this study, were fairly robust to the presence of null alleles in that data set. The largest source of pedigree error occurred when alleles were incorrectly scored by either one repeat unit above or below the

actual size. This latter problem is much reduced on the latest generation of capillary DNA analysis machines.

Li et al. (2003) discuss the importance of testing the inheritance patterns of microsatellite loci before they are used in population genetic or parentage analyses. They found significant deviations from Mendelian expectations among several loci in the Pacific abalone, *Haliotis discus hannai*. In their study, observed deviations from Mendelian expectations likely resulted from the presence of null alleles, which occur when there are mutations in the primer site surrounding the tandem repeats (for a review see Dakin and Avise 2004). Null alleles do not amplify during PCR resulting in an increase in the apparent frequency of homozygotes in the population (Kalinowski and Taper 2006).

I tested for segregation distortion in three reconstructed families and observed instances where the ratio of alleles showed significant deviation from Mendelian expectations. Given that reconstructed families were used for this analysis, it is possible that the observed deviations from Mendelian expectations are the result of errors in the pedigree; families containing individuals placed in the wrong full-sib group would cause deviations from the expected Mendelian ratio of alleles. However, the same two loci were affected in both families where the distortion was observed, which suggests that observed deviations from Mendelian expectations may exist among these loci, and are not simply the result of pedigree error.

Implications for Conservation

Due to increased levels of habitat loss and species decline, captive breeding may be the only method to prevent the extinction of many critically endangered species (Theodorou and Couvet 2004; Yue et al. 2004). However, if not managed properly, captive breeding programs may actually aid in the genetic deterioration of threatened species (Woodworth et al. 2002). This is especially apparent in this example of a broadcast spawning species where the census population size may be orders of magnitude greater than the effective population size. Given that an increase in abundance of *H. kamtschatkana* does not necessarily increase the genetic diversity, proper management is essential to prevent genetic deterioration.

The most common management recommendations for slowing the rate of genetic deterioration in captivity involve the equalization of family sizes (Fernandez and Caballero 2001; Woodworth et al. 2002), the maintenance of small fragmented captive populations instead of a single larger population (Margan et al. 1998; Fiumera et al. 2000; Woodworth et al. 2002), frequent migration of wild individuals to the captive program (Woodworth et al. 2002; Theodorou and Couvet 2004), and if possible the use of controlled crosses carried out with pedigree information (Senner 1980). These recommendations are all aimed at increasing the effective population size of the breeding program and thus slowing the impact of genetic drift and inbreeding depression. However, it is important to realize that for small declining populations, inbreeding and the loss of genetic diversity are unavoidable (Frankham 2005); the goal of a captive

breeding program is to slow these factors as much as possible until the animals can be reintroduced to the wild.

The success of a captive breeding program is also dependant on adequate monitoring after captive-bred animals have been released to the wild (Nicoll et al. 2004). Since BHCAP began supplementing the wild population with captive-bred pinto abalone, they have been monitoring species abundance in and around supplemented sites. No studies have assessed the impact of out-planting captive-bred pinto abalone on the genetic diversity of the wild population. This is especially important given the potential for captive-bred offspring to swamp locally adapted genotypes in areas were they are being introduced to the wild (Smith and Conroy 1992). I recommend that field studies should be carried out in areas where large numbers of captive-bred abalone have been released to assess the impact, if any, that mass introduction is having on wild genetic diversity. If the number of captive-bred individuals is too large, there is the possibility of altering the genetic structure or local adaptation of the wild population (Smith and Conroy 1992). However, I predict that the reintroduction of individuals pooled from all three spawning groups, combined with the relatively high heterozygosity in the captive-bred pinto abalone may slow any negative effects of captive-breeding on the genetic structure of the wild pinto abalone population.

The captive breeding program for *H. kamtschatkana* is also developing a commercial aspect where BHCAP will sell some abalone to offset the cost of spawning and out-planting. BHCAP is also investigating the use of a selective

breeding program to maximize commercially desirable traits such as growth rate and body size. This poses a problem for conservation; the goal of the out-planting program is to reintroduce animals without causing any changes to their genetic structure, whereas the commercial program will actively strive for 'genetic improvement'. It would then be necessary for captive breeders to maintain two distinct captive lineages and apply different management strategies for each. It may be possible to sell the abalone destined for re-introduction, but it would be detrimental to re-introduce abalone to the wild that had undergone artificial selection for the marketplace. Whether the abalone are bred to aid conservation or for commercial sale, the maintenance of genetic diversity is essential to facilitate both natural selection in the wild and selective breeding in aquaculture (Smith and Conroy 1992; Boudry et al. 2002).

Franklin (1980) brings up the interesting point that conservation biologists decide whether to preserve an exact phenotype or rather the phylogenetic line that allows for continued evolutionary change. He uses the example of either preserving 'the elephant' or alternately ensuring the survival of all 'elephant-like descendants'. The strategy of the conservation program will be very different for these two possibilities. However, the amount of genetic diversity required to sustain the long-term fitness and evolutionary potential of a population is unknown (Gautschi et al. 2003).

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Table 1

The four sample groups of pinto abalone analyzed in this study consist of one wild population and three captive-spawned groups. Captive breeding records were used to determine the minimum number of wild males and females that could have contributed gametes to each spawning group. Brackets indicate the number of these broodstock for which DNA samples were still available at the time of my study. Information about the age or relatedness of the wild abalone broodstock is unknown.

Group	Year of Spawn	Potential fathers	Potential mothers	Number of offspring sampled	Number of offspring genotyped
Wild	-	-	-	133	106
B-group*	2002	6 (2)	7 (4)	192	179
R-group	2002	3 (1)	3 (1)	253	215
T-group	2003	5 (3)	3 (2)	200	154

*The B-group was composed of offspring from two separate spawnings that were pooled together: The first spawning had three potential fathers and three potential mothers, the second spawning had three potential fathers and four potential mothers. As a result, there are 21 possible full-sib families in this group.

Table 2

A description of the microsatellite loci screened for use in this study. Annealing temperature, size range, and number of alleles are from this study and vary slightly from those published in Miller et al. 2001. Given the magnitude of their sample size, Miller et al. (2001) generally found more rare alleles and had a slightly greater size range of alleles present.

Locus	Repeat	Primer sequences	Annealing temp.	Size (bp)	No. of alleles
Hka12	Dinucleotide	F: tgatgcatggtaaatcactta	48°C	NA	NA
		R: gtttcagtatgaagagggtctaat			
HKa37	Tri/tet compound	F: gtttctctgaacaaagtgactaac	50°C	243-292	12
		R: ggtgtggttctaaaaatag			
Hka40	Dinucleotide	F:ccctaaacagatattgacaat	47°C	115-163	22
		R:ctagcaatattcaaaattacaag			
Hka43	tetranucleotide	F:cgcaacatgtctacatata	48°C	182-294	15
		R:gtttggtctgtacattttatctatgt			
Hka48	Dinucleotide	F:cgttagtggggaaagata	48°C	NA	NA
		R:gcgtgatttgttttgaccaa			
Hka56	Dinucleotide	F:cgataatcgtgtggtaaga	50°C	97-151	25
		R:gtttgtggcatggtatctcatt			
Hka65	Dinucleotide (imperfect)	F:gggttcaattggacaca	50°C	118-201	28
		R:acggttgtaaagtacgttata			

Table 3

Optimized parameters for determining full-sib relationships among captive-bred pinto abalone using the program Pedigree 2.2 (Smith et al. 2001). Pedigree reconstruction was carried out for three different captive-bred groups of abalone.

Group	Iterations	Full-sib	Temperature	Weight	Seed
B-Group	5000000	On	30	5	Random
R-Group	5000000	On	30	5	Random
T-Group	5000000	On	30	5	Random

Table 4

List cytochrome c oxidase subunit 1 (CO1) haplotypes present among 144 wild pinto abalone. All sequences are aligned to nucleotide position 90 in the full AF060845 sequence (Metz et al. 1998).

		Position of variable sites											
	n	129	141	222	249	270	339	342	352	379	384	477	499
1*	50	C	A	A	G	G	A	A	C	T	G	C	C
2*	37	T	.	G	.	.	.	.	.	.	.	.	.
3*	21	T	G	G	.	.	.	.	.	.	.	.	.
4	14	T	.	G	.	.	.	.	.	.	A	.	.
5	5	.	.	.	.	.	.	G	.	.	.	.	.
6	5	.	.	.	.	.	.	.	.	.	.	T	.
7	4	T	.	G	.	.	G	.	.	.	.	.	.
8	3	T	.	G	.	A	.	.	.	.	.	.	.
9	1	.	.	.	.	.	.	.	.	.	.	.	T
10	1	.	.	.	.	.	.	.	.	.	A	.	.
11	1	T	.	.	.	.	.	.	T	.	.	.	.
12	1	T	.	.	.	.	.	.	.	.	.	.	.
13	1	T	.	G	C	.	.	.	.	C	.	.	.

* Haplotypes that were also present among captive-bred samples

Table 5

Population diversity measures for three captive-bred and one wild population of pinto abalone based on five polymorphic microsatellite loci. This table includes expected (H_E) and observed (H_O) heterozygosity, inbreeding coefficient (F_{IS}), as well as the number of alleles (N_A), and allelic richness (R_s) standardized to 60 diploid individuals.

		Locus					
Sample		Hka43	Hka56	Hka65	Hka37	Hka40	All
Wild	H_O	0.951	0.915	0.881	0.750	0.956	0.891
n = 106	H_E	0.884	0.945	0.916	0.716	0.917	0.876
	F_{IS}	-0.077	0.032	0.039	-0.048	-0.043	-0.017
	N_A	15	25	28	12	19	19.8
	R_s	14.1	23.9	25.7	12.0	18.2	18.8
B-group	H_O	0.771	0.862	0.804	0.780	0.772	0.798
(hatchery)	H_E	0.714	0.798	0.722	0.626	0.765	0.725
n = 179	F_{IS}	-0.080*	-0.080*	-0.110*	-0.246*	-0.007*	-0.099*
	N_A	8	11	10	5	12	9.2
	R_s	7.4	9.7	9.1	4.8	10.8	8.4
R-group	H_O	0.957	0.730	0.892	0.516	0.946	0.808
(hatchery)	H_E	0.805	0.724	0.791	0.430	0.835	0.717
n = 214	F_{IS}	-0.187*	-0.008*	-0.127*	-0.200*	-.0130*	-0.126*
	N_A	7	6	9	5	12	7.8
	R_s	7.0	6.0	8.6	5.0	11.0	7.5
T-group	H_O	0.748	0.937	0.847	0.806	0.799	0.827
(hatchery)	H_E	0.708	0.815	0.732	0.637	0.727	0.727
n = 154	F_{IS}	-0.063*	-0.145*	-0.148*	-0.265*	-0.088*	-0.139*
	N_A	8	9	9	5	11	8.4
	R_s	7.9	8.8	8.5	4.7	10.6	8.1

* Significant deviation from zero after sequential Bonferroni correction for multiple tests.

Table 6

Pairwise F_{ST} values (Weir and Cockerhams Θ) for all three captive-bred groups and the wild population of pinto abalone sampled in this study. All are highly significant ($p<0.0001$).

	T-group	B-group	R-group	wild
T-group	-	-	-	-
B-group	0.048	-	-	-
R-group	0.149	0.130	-	-
Wild	0.102	0.097	0.077	-

Table 7

P-values for all exact tests of population differentiation between all three captive-bred groups and the wild population of pinto abalone sampled in this study.

	T-group	B-group	R-group	wild
T-group	-	-	-	-
B-group	0.003*	-	-	-
R-group	0.007*	0.003*	-	-
Wild	0.113	0.025	0.036	-

*significant after Bonferroni correction for multiple tests.

Table 8

Results from the pedigree reconstruction for each captive-bred group of pinto abalone. This includes the number of full-sib families in each group, the number of offspring from each family, and the common cytochrome c oxidase subunit 1(CO1) haplotype among sampled offspring from each family. The parental reconstruction function in Pedigree 2.2 was combined with CO1 haplotypes and microsatellite genotypes of known parents to elucidate parentage of each family. Reconstructed parents that did not match any sampled broodstock were given alphabetical labels.

Group	Family number	Number of offspring (n)	CO1 Haplotype	Father	Mother
B-group	1	108	3	453	613
	2	19	3	B	613
	3	6	1	623	T
	4	6	1	453	T
	5	5	2	D	Y
R-group	1	87	1	623	T
	2	44	2	623	610
	3	24	2	623	611
	4	24	1	623	443
T-group	1	78	3	A	Z
	2	19	3	941	Z
	3	12	1	E	X
	4	8	3	A	X
	5	5	3	F	S

Figure 1

Distribution of full-sib family sizes for each of the three captive-bred groups of pinto abalone as reconstructed using Pedigree 2.2.

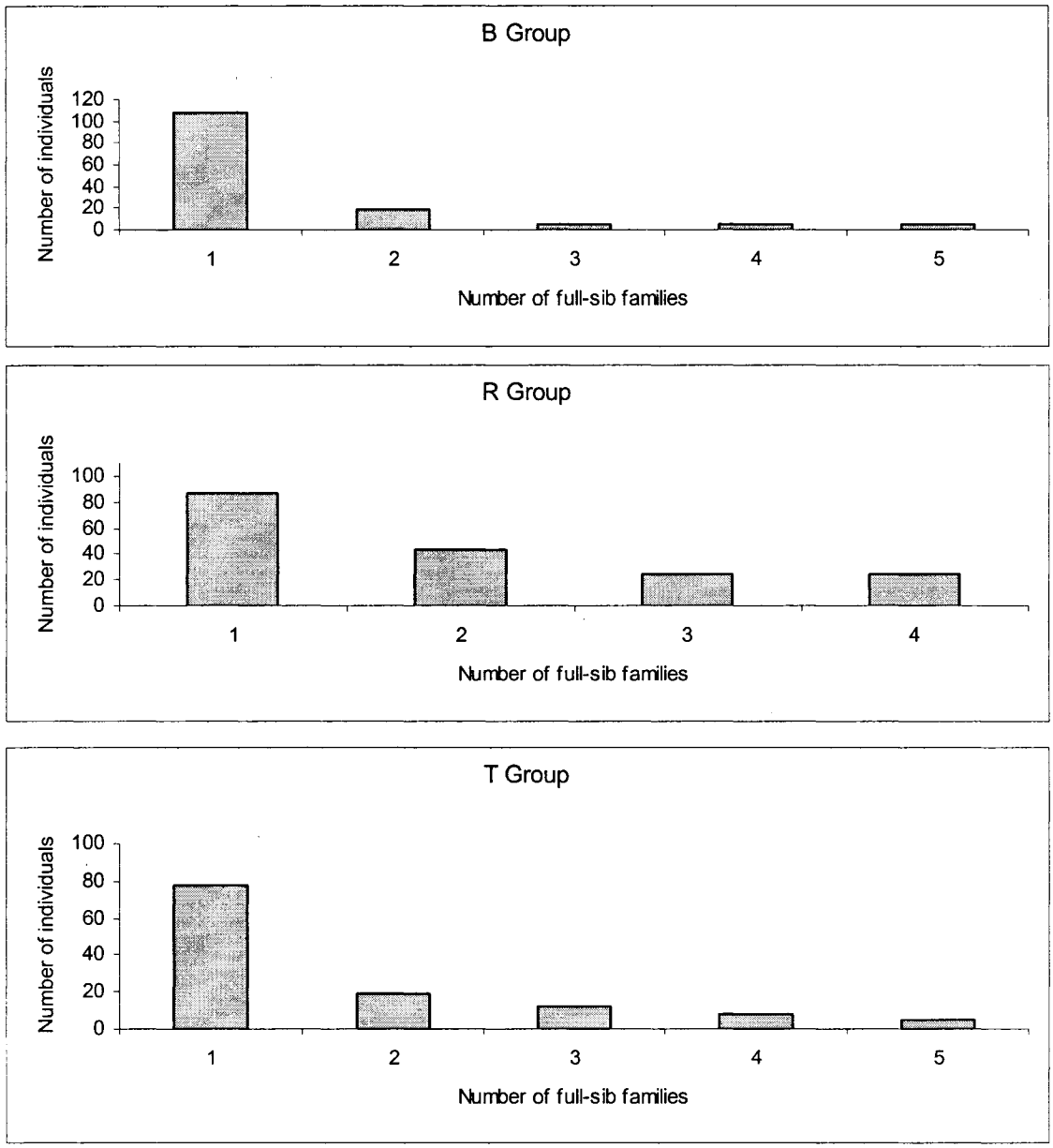
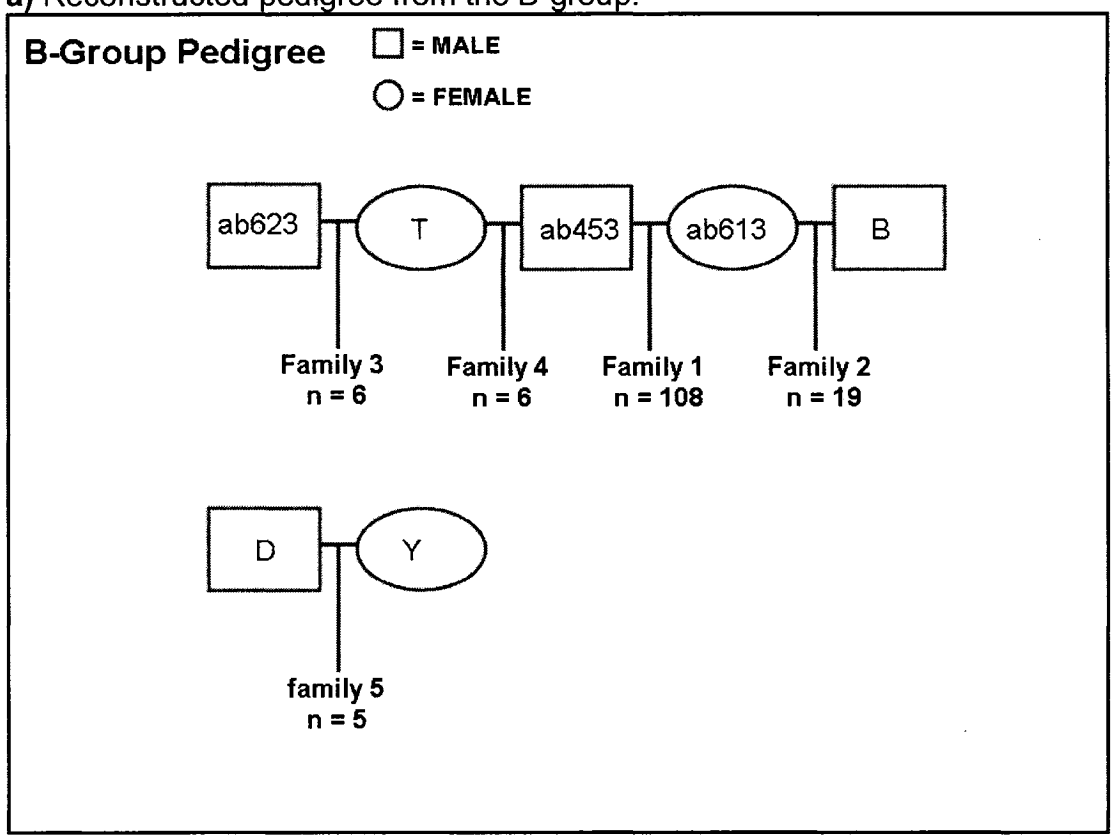


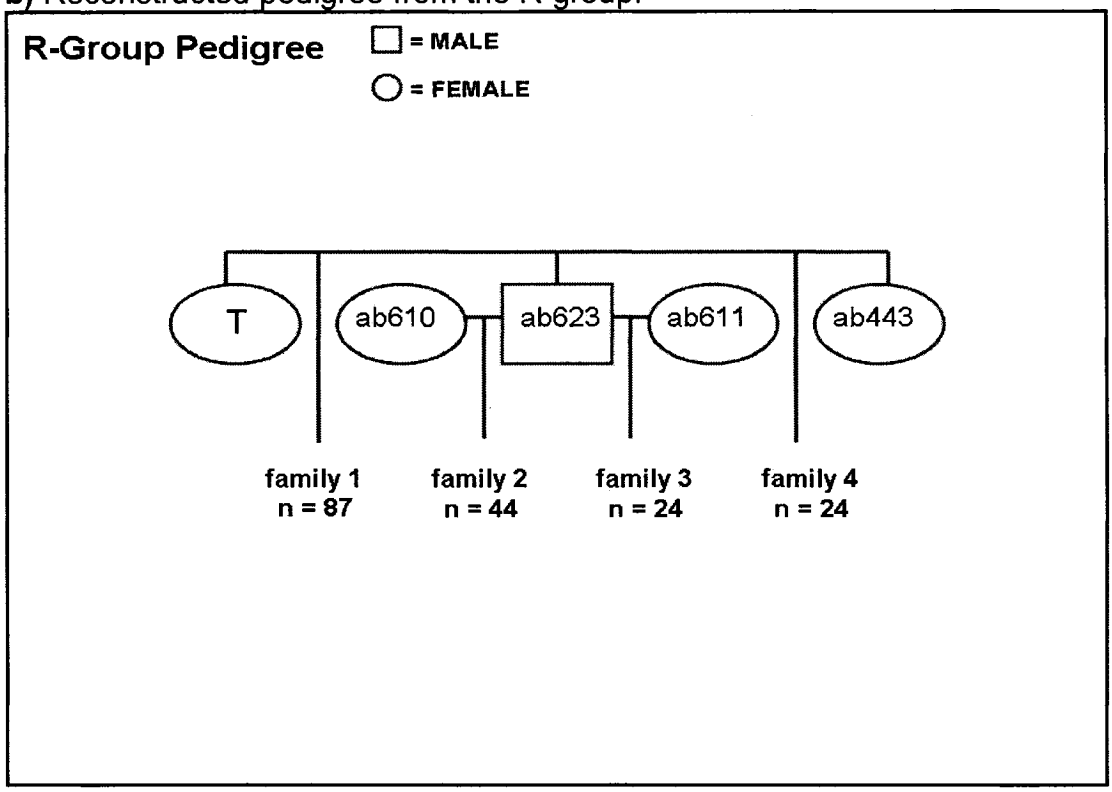
Figure 2 (a-c)

Reconstructed pedigree for each of the three captive-bred groups of pinto abalone: Pedigree 2.2 was used to reconstruct parental genotypes at all five loci; these were compared to the genotypes of broodstock for which we had DNA samples (indicated by abalone ID numbers). Reconstructed parental genotypes that did not match any sampled broodstock were given alphabetic labels.

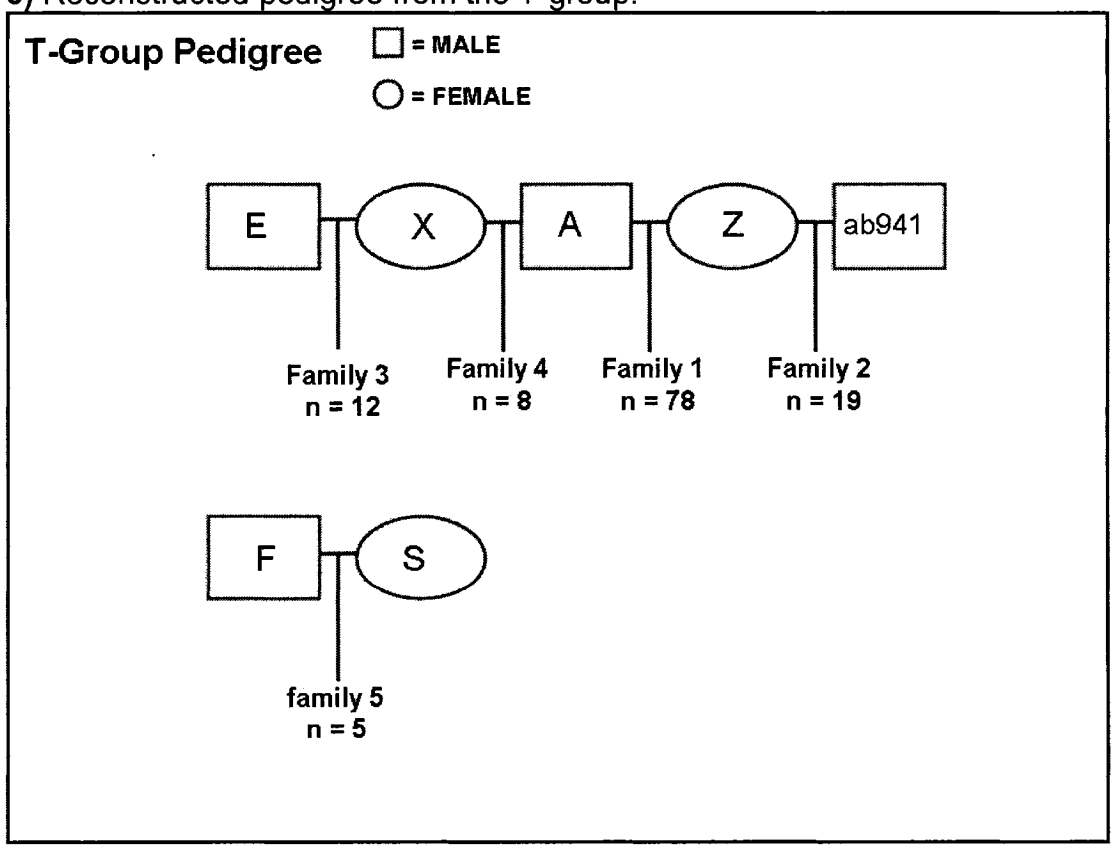
a) Reconstructed pedigree from the B-group.



b) Reconstructed pedigree from the R-group.



c) Reconstructed pedigree from the T-group.



Appendix A

Population allele frequencies at each locus among the four sampled groups of pinto abalone. Alleles are presented as the number of base-pairs and frequency is percentage of total.

Locus	Allele (base-pairs)	T-group (n=154)	B-group (n=179)	R-group (n=215)	Wild (n=106)
Hka43	182	2.16	-	-	2.43
	190	-	-	-	1.46
	194	18.71	18.82	25.96	2.91
	198	17.27	22.65	6.49	15.53
	202	-	6.76	15.87	16.99
	206	-	0.88	5.29	5.34
	210	2.88	2.94	27.4	8.74
	214	7.19	2.94	-	16.02
	218	-	0.88	4.81	14.56
	222	47.12	44.12	14.18	8.25
	226	2.16	-	-	3.88
	230	2.52	-	-	0.97
	234	-	-	-	1.46
	246	-	-	-	0.97
	294	-	-	-	0.49
Hka56	97	13.99	19.31	-	1.22
	99	4.20	4.48	40.81	10.98
	101	-	-	-	7.93
	103	27.62	27.59	-	0.61
	105	-	0.69	-	1.83
	107	-	1.38	-	7.32
	111	17.13	23.45	13.24	7.32
	113	-	2.07	11.89	4.27
	115	-	0.69	-	2.44
	117	1.05	-	-	1.22
	119	4.90	1.38	4.05	3.05
	121	-	-	-	8.54
	123	23.43	18.28	27.84	6.10
	125	-	0.69	-	4.88
	127	-	-	-	5.49
	129	-	-	2.16	6.71
	131	-	-	-	3.66
	133	-	-	-	6.10
	135	-	-	-	1.22
	137	-	-	-	1.22
	141	5.59	-	-	2.44
	143	-	-	-	0.61
	145	-	-	-	2.44
	149	2.10	-	-	1.83
	151	-	-	-	0.61
Hka65	116	-	-	-	0.60
	122	25.85	19.63	-	20.83
	124	40.68	39.26	26.20	9.52
	126	0.85	-	-	4.17
	128	-	-	-	1.19
	130	-	2.45	20.48	5.95
	132	-	1.84	3.31	2.98

	134	-	-	-	0.60
	156	-	-	-	0.60
	158	-	2.15	-	1.19
	162	-	-	-	0.60
	164	-	-	-	2.38
	166	-	-	0.90	0.60
	168	2.54	0.61	2.11	1.19
	170	-	-	-	1.19
	173	5.51	1.23	6.93	11.31
	175	1.27	2.45	9.04	4.76
	177	18.22	29.14	29.22	9.52
	179	3.81	-	-	1.79
	181	-	-	-	3.57
	183	-	-	-	2.38
	185	-	1.23	-	3.57
	187	-	-	-	1.19
	189	1.27	-	1.81	2.38
	191	-	-	-	2.98
	197	-	-	-	1.19
	199	-	-	-	1.19
	201	-	-	-	0.60
Hka37	243	14.81	12.99	3.18	4.17
	246	41.20	48.03	73.57	38.33
	250	-	-	2.55	5
	254	41.67	35.83	16.24	36.67
	257	0.93	-	4.46	4.17
	261	-	1.18	-	1.67
	268	-	-	-	0.83
	272	1.39	-	-	0.83
	275	-	1.97	-	2.50
	278	-	-	-	3.33
	282	-	-	-	0.83
	292	-	-	-	1.67
Hka40	115	-	0.90	2.70	-
	117	-	-	-	1.10
	119	1.12	-	-	3.30
	123	-	-	-	2.20
	127	-	18.56	-	-
	129	-	3.29	15.41	0.55
	131	25.75	40.12	-	13.74
	133	4.10	17.96	30.27	15.93
	135	43.66	4.19	-	8.79
	137	11.57	6.29	12.43	4.95
	139	-	-	-	2.20
	141	2.99	-	-	8.24
	143	1.49	-	-	2.20
	147	-	-	-	0.55
	149	-	2.10	9.46	-
	151	1.87	-	1.08	2.75
	153	2.24	3.89	14.05	4.40
	155	-	0.60	1.89	2.75
	157	3.73	-	3.78	3.85
	159	-	1.50	6.49	7.14
	161	-	-	-	4.40
	163	1.49	0.60	2.16	10.99

Appendix B

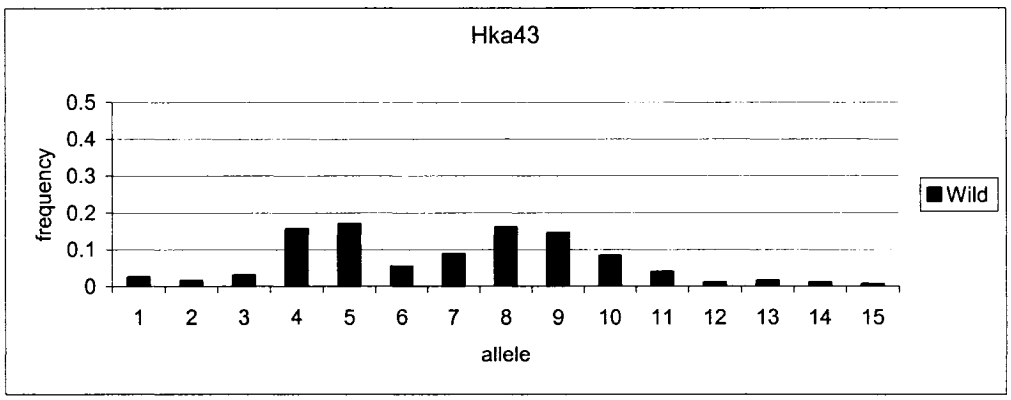
Pairwise analysis of linkage disequilibrium for each locus in each sample group of pinto abalone.

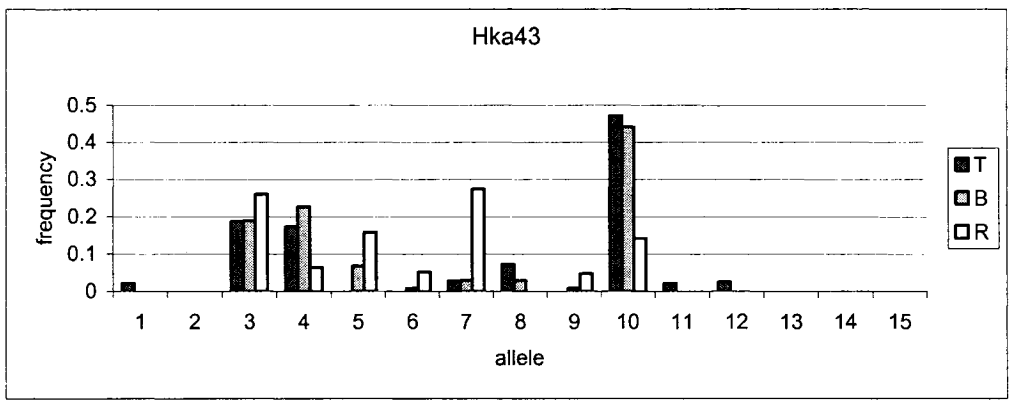
Population	Locus 1	Locus 2	P-value
T-group	Hka43	Hka56	<0.0001*
	Hka43	Hka65	<0.0001*
	Hka56	Hka65	<0.0001*
	Hka43	Hka37	0.04344
	Hka56	Hka37	0.0151
	Hka65	Hka37	<0.0001*
	Hka43	Hka40	<0.0001*
	Hka56	Hka40	<0.0001*
	Hka65	Hka40	<0.0001*
	Hka37	Hka40	0.5178
B-group	Hka43	Hka56	<0.0001*
	Hka43	Hka65	<0.0001*
	Hka56	Hka65	<0.0001*
	Hka43	Hka37	0.00286*
	Hka56	Hka37	<0.0001*
	Hka65	Hka37	0.7698
	Hka43	Hka40	<0.0001*
	Hka56	Hka40	<0.0001*
	Hka65	Hka40	<0.0001*
	Hka37	Hka40	<0.0001*
R-group	Hka43	Hka56	<0.0001*
	Hka43	Hka65	<0.0001*
	Hka56	Hka65	<0.0001*
	Hka43	Hka37	<0.0001*
	Hka56	Hka37	<0.0001*
	Hka65	Hka37	<0.0001*
	Hka43	Hka40	<0.0001*
	Hka56	Hka40	<0.0001*
	Hka65	Hka40	<0.0001*
	Hka37	Hka40	<0.0001*
Wild	Hka43	Hka56	1.000
	Hka43	Hka65	0.5595
	Hka56	Hka65	0.2921
	Hka43	Hka37	0.1610
	Hka56	Hka37	1.000
	Hka65	Hka37	0.3585
	Hka43	Hka40	1.000
	Hka56	Hka40	1.000
	Hka65	Hka40	0.2459
	Hka37	Hka40	0.6211

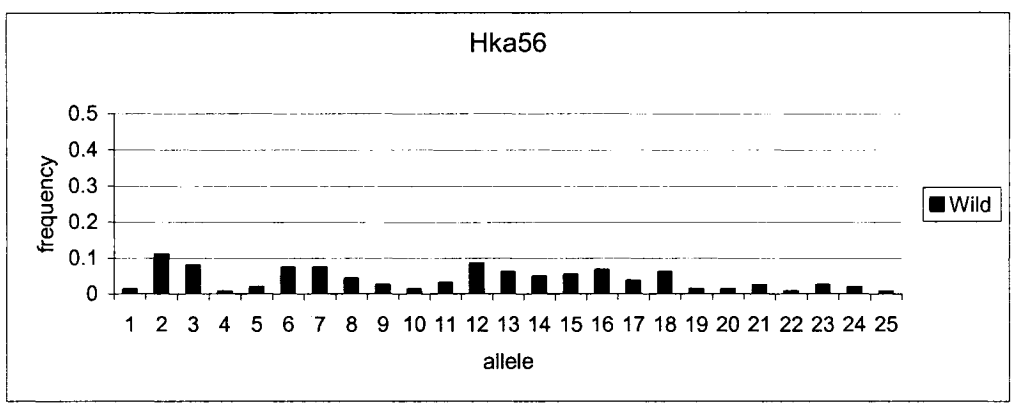
* Indicates significant deviation from linkage equilibrium after sequential Bonferroni correction.

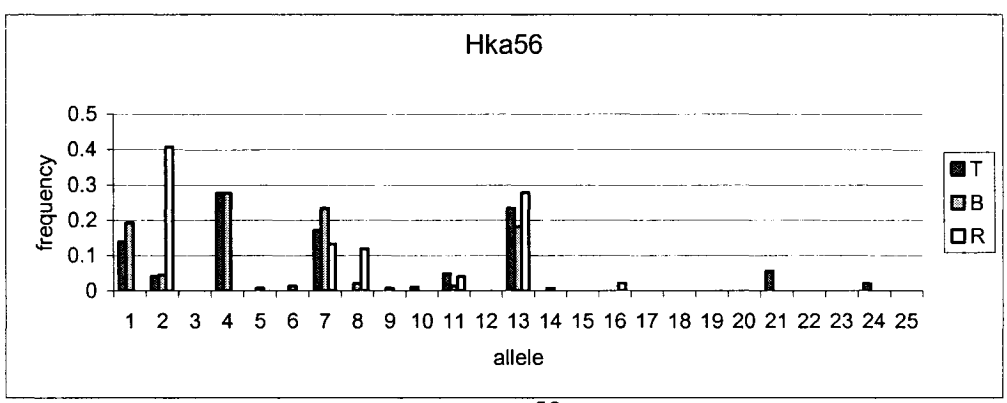
Appendix C

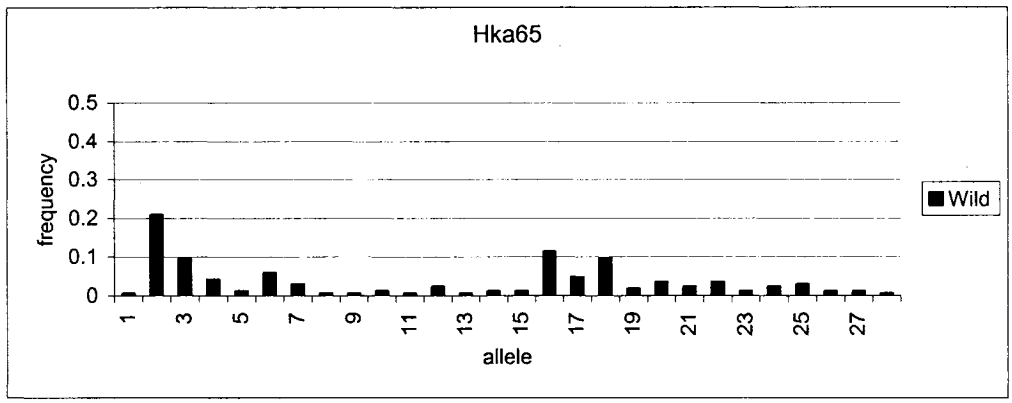
Gene frequency changes from wild to captive-bred groups at each of five microsatellite loci (Hka43, Hka56, Hka65, Hka37, and Hka40).







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0.1
0
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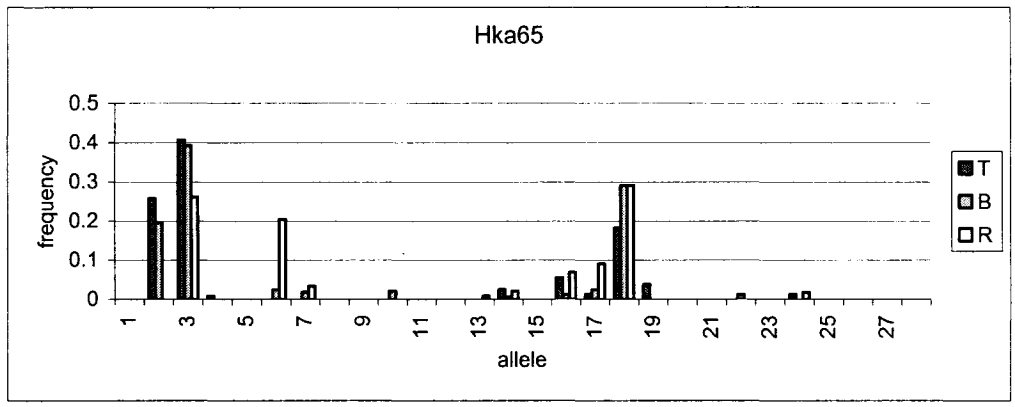
allele
Wild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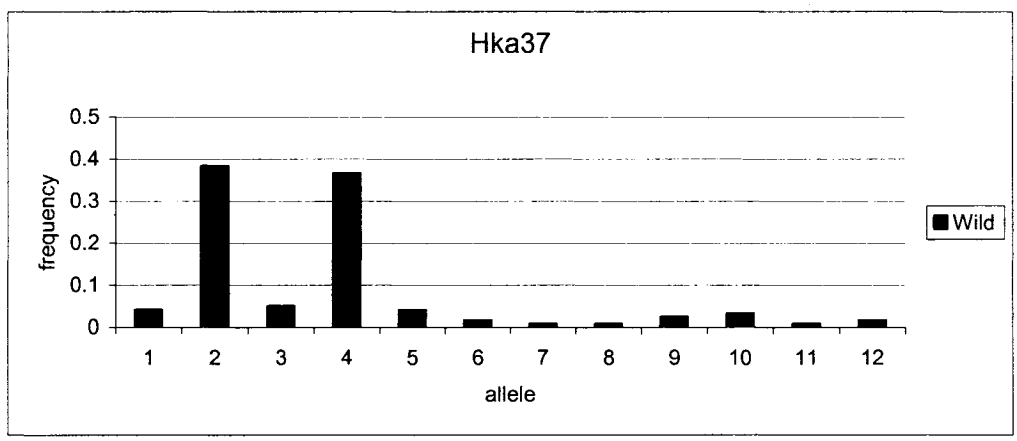
B
R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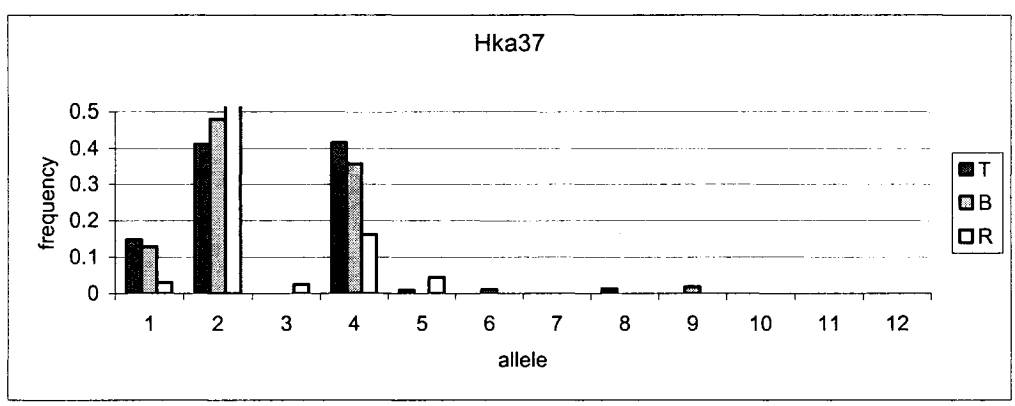
allele
Wild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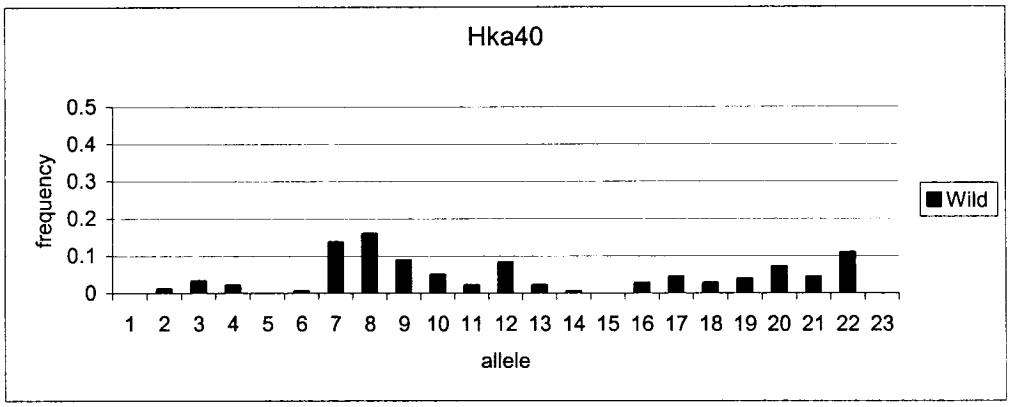
allele
T
B
R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0.1
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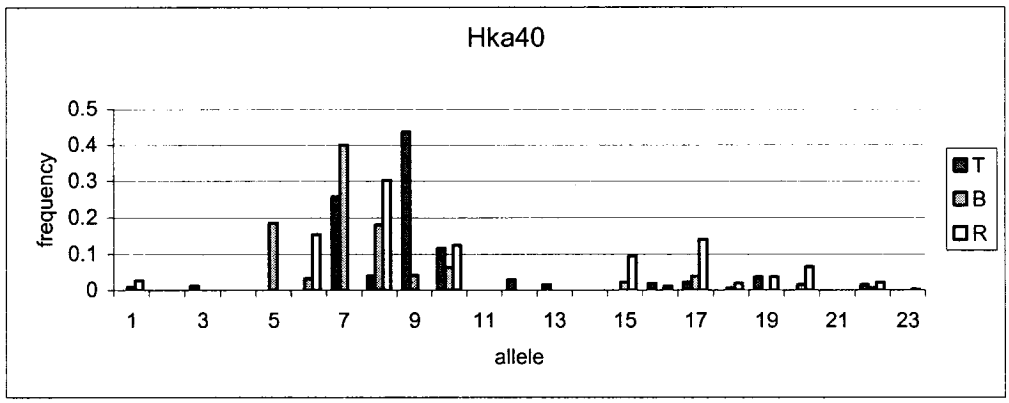
allele
Wild


Hka40
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0.4
0.3
0.2
0.1
0
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allele
T
B
R

Appendix D

Specific offspring present in each reconstructed full-sib family of pinto abalone. Offspring ID numbers correspond with tags affixed to the shells of juvenile abalone; parents of each family are indicated in parentheses.

Family 1 (♂453 x ♀613):

B2-33G	B2-35G	B1-47G	B1-48G	B1-49G	B1-54G	B1-56G	B1-57G	B1-59G
B6-60G	B6-64G	B6-72G	B6-74G	B6-77G	B6-78G	B6-79G	B6-80G	B5-87G
B5-85G	B5-90G	B5-92G	B5-93G	B5-01B	B5-02B	B5-03B	B5-05B	B7-12B
B7-13B	B7-14B	B7-15B	B7-18B	B7-19B	B7-20B	B7-22B	B3-27B	B3-32B
B2-50B	B2-55B	B2-57B	B2-63B	B2-65B	B6-79B	B6-80B	B6-83B	B6-84B
B1-89B	B1-90B	B1-96B	B1-97B	B1-04O	B5-20O	B5-21O	B5-23O	B5-25O
B5-27O	B7-35O	B7-37O	B7-39O	B2-38G	B2-41G	B1-46G	B1-55G	B7-44O
B1-58G	B6-63G	B6-67G	B6-68G	B6-75G	B6-76G	B6-81G	B5-82G	B5-83G
B5-85G	B5-86G	B5-96G	B5-97G	B7-08B	B7-10B	B7-16B	B7-24B	B7-26B
B3-38B	B3-40B	B3-42B	B3-43B	B2-49B	B2-52B	B2-53B	B2-56B	B2-60B
B2-51B	B6-68B	B6-70B	B6-71B	B6-72B	B6-74B	B6-76B	B6-78B	B6-81B
B6-82B	B1-92B	B1-93B	B1-03O	B1-07O	B1-08O	B5-22O	B7-42O	B7-43O

Family 2 (623 x T):

B1-42G	B5-94G	B7-23B	B3-44B	B6-67B	B5-31O

R5-27O	R6-42Y	R5-28O	R3-03O	R6-41Y	R3-04O	R8-18Y	R3-18O	R4-06Y
R6-43Y	R3-06O	R5-32O	R4-07Y	R3-07O	R8-21Y	R5-21O	R8-22Y	R5-22O
R8-23Y	R6-47Y	R2-94Y	R6-37Y	R6-49Y	R7-55Y	R7-57Y	R7-58Y	R8-62Y
R8-65Y	R8-66Y	R8-72Y	R8-74Y	R8-75Y	R2-77Y	R2-79Y	R2-82Y	R4-66B
R8-67B	R2-73B	R2-75B	R2-76B	R5-77B	R5-80B	R5-81B	R3-82B	R3-83B
R3-86B	R7-91B	R6-95B	R4-22G	R4-26G	R8-27G	R8-28G	R2-34G	R2-36G
R5-40G	R7-47G	R7-48G	R7-49G	R7-51G	R4-59G	R4-58G	R-64G	R-53B
R-19B	R-02B	R-20B	R-32B	R-48B	R2-99Y	R3-13O	R7-15Y	R3-16O
R6-42Y	R8-69B	R7-87B	R4-24G	R3-43G	R-47B	R-06B	R2-72B	R-37B
R-62B	R-34B	R-23B	R-36B	R4-30Y	R-29B			

Family 3 (A x Z):

T-89O	T-92O	T-26B	T-33W	T-22G	T-42G	T-B094	T-22B	T-02B
T-77O	T-57G	T-47W	T-69O	T-68O	T-97O	T-17B	T-72O	T-B087
T-84O	T-80O	T-39B	T-15B	T-87O	T-14B	T-81O	T-09B	T-14O
T-06B	T-34B	T-12B	T-95O	T-86O	T-41B	T-65W	T-68W	T-01W
T-57W	T-34W	T-19W	T-31W	T-08W	T-40W	T-B090	T-72W	T-11G
T-36G	T-04W	T-59W	T-52W	T-39W	T-37W	T-23W	T-22W	T-29G
T-69W	T-17W	T-77W	T-20W	T-16W	T-41W	T-62G	T-B097	T-01G
T-16G	T-23G	T-13G	T-14G	T-67O	T-71O	T-36B	T-79O	T-28B
T-37B	T-21B	T-91O	T-75W	T-11B	T-11W	T-61G	T-78O	T-83O
T-40B	T-42B	T-20B	T-44W					

Family 4 (623 x 610):

R7-14Y	R4-26Y	R6-38Y	R3-14O	R3-15O	R2-86Y	R5-29O	R5-30O	R2-88Y
R3-05O	R5-31O	R6-45Y	R6-10Y	R4-34Y	R6-46Y	R3-10O	R5-23O	R6-12Y
R8-24Y	R6-36Y	R5-24O	R4-25Y	R7-54Y	R8-71Y	R6-93B	R6-94B	R6-97B
R8-29G	R8-30G	R8-31G	R2-35G	R5-37G	R5-41G	R3-44G	R3-46G	R-52G
R6-48Y	R-11B	R-40B	R-43B	R-30B	R-31B	R-50B	R-52B	

Family 5 (623 x 611):

R3-01O	R6-40Y	R7-17Y	R4-29Y	R3-17O	R7-19Y	R4-32Y	R6-44Y	R3-12O
R5-37O	R7-53Y	R8-73Y	R2-80Y	R5-79B	R7-90B	R3-45G	R7-50G	R-63G
R7-16Y	R5-33O	R4-33Y	R4-35Y	R-26B	R-46B			

Family 6 (B x 613):

B1-53G	B1-61G	B6-66G	B7-07B	B2-58B	B2-64B	B6-73B	B6-87B	B5-28O
B5-29O	B5-30O	B2-39G	B1-50G	B2-62B	B2-66B	B1-01O	B1-10O	B5-15O
B7-45O								

Family 7 (623 x 443):

R2-85Y	R3-11O	R7-56Y	R7-59Y	R8-69Y	R2-76Y	R2-81Y	R4-65B	R5-78B
R3-85B	R7-88B	R5-39G	R3-42G	R4-57G	R-62G	R-38B	R-54B	R-55B
R-56B	R-01B	R-61B	R-63B	R-22B	R-14B			

Family 8 (941 x Z):

T-61W	T-12G	T-04B	T-62W	T-47G	T-06G	T-49W	T-28G	T-B085
T-59G	T-76O	T-96O	T-90O	T-13B	T-23B	T-54G	T-70W	T-38W
T-36W								

Family 9 (A x X):

T-82O	T-33B	T-70O	T-75O	T-66O	T-58G	T-66W	T-53W

Family 10 (E x X):

T-45W	T-B091	T-B093	T-32W	T-10W	T-19G	T-20G	T-B092	T-18G
T-28W	T-21G	T-B096						

Family 11 (453 x T):

B7-06B	B3-30B	B3-41B	B3-45B	B7-38O	B7-41O

Family 12 (D x Y):

B2-37G	B7-17B	B7-25B	B1-91B	B5-17O

Family 13 (F x S):

T-08B	T-39G	T-27G	T-48G

Appendix E

Results from the analysis for linkage among the five microsatellite loci used in theis study. Analysis was carried out using parent and sibling genotypes from three reconstructed full-sib groups of captive-bred pinto abalone and the program LINKMFEX (Danzmann 2005). This table lists the recombination values for each pairwise comparison of loci and their associated LOD scores.

Family	1st locus	2nd locus	Recombination (r)	LOD score	n
623 x T	HKa43	Hka56	0.41	0.40	54
	HKa43	Hka65	0.41	0.26	41
	HKa43	Hka37	0.29	1.51	38
	HKa43	Hka40	0.48	0.02	44
	Hka56	Hka65	0.44	0.13	41
	Hka56	Hka37	0.45	0.10	38
	Hka56	Hka40	0.41	0.32	44
	Hka65	Hka37	0.30	1.21	36
	Hka65	Hka40	0.46	0.05	39
	Hka37	Hka40	0.41	0.26	41
623 x 610	HKa43	Hka56	0.28	1.09	25
	HKa43	Hka65	0.45	0.06	31
	HKa43	Hka37	0.42	0.15	24
	HKa43	Hka40	0.42	0.15	24
	Hka56	Hka65	0.39	0.24	23
	Hka56	Hka37	0.44	0.05	18
	Hka56	Hka40	0.42	0.10	19
	Hka65	Hka37	0.38	0.26	21
	Hka65	Hka40	0.50	0	22
	Hka37	Hka40	0.50	0	18
453 x 613	HKa43	Hka56	0.41	0.58	85
	HKa43	Hka65	0.46	0.15	92
	HKa43	Hka37	0.50	0	68
	HKa43	Hka40	0.33	2.21	90
	Hka56	Hka65	0.47	0.06	85
	Hka56	Hka37	0.34	1.50	65
	Hka56	Hka40	0.40	0.76	83
	Hka65	Hka37	0.42	0.44	72
	Hka65	Hka40	0.41	0.66	95
	Hka37	Hka40	0.49	0.01	75

Appendix F

Test for segregation distortion among the five microsatellite loci used in this study. Log-likelihood ratio test for goodness of fit to Mendelian expectations is presented for three reconstructed full-sib partitions of captive-bred pinto abalone.

Family	Loci	G-value	n
623 x T	Hka43	0.46	55
	Hka56	0.16	55
	Hka65	12.26*	48
	Hka37	28.15*	46
	Hka40	0.08	52
623 x 610	Hka43	0.26	35
	Hka56	2.50	26
	Hka65	0.13	32
	Hka37	1.01	25
	Hka40	0.04	25
453 x 613	Hka43	3.32	98
	Hka56	9.40*	91
	Hka65	5.18*	103
	Hka37	20.70*	77
	Hka40	0.01	101

* significant deviation from Mendelian expectations.

Appendix G

Cytochrome c oxidase subunit 1 (CO1) haplotypes used to corroborate reconstructed pedigree among each captive-bred group of pinto abalone.

Spawning group	Family	Offspring ID	Haplotype
B-Group	1	B2-35G	3
		B5-20O	3
		B1-89B	3
		B6-67G	3
	2	B2-39G	3
		B1-53G	3
		B2-64B	3
		B5-28O	3
	3	B1-42G	1
		B5-94G	1
		B3-44B	1
		B6-67B	1
	4	B7-06B	1
		B3-30B	1
		B3-41B	1
		B3-45B	1
	5	B2-37G	2
		B7-17B	2
		B7-25B	2
		B1-91B*	1
R-Group	1	R4-06Y	1
		R2-79Y	1
		R8-28G	1
		R2-99Y	1
		R2-72B	1
		R-37B	1
		R-62B	1
		R-34B	1
	2	R5-29O	2
		R6-46Y	2
		R4-25Y	2
		R-30B	2
	3	R6-40Y	2
		R5-33O	2
		R2-80Y*	1
	4	R2-85Y	1
		R5-78B	1
		R3-85B	1
		R7-88B	1
		R5-39G*	3
		R-38B	1
		R-54B	1
		R-61B	1
		R-22B	1

T-Group	1	T-14O	3
		T-06B	3
		T-34W	3
		T-02B	3
		T-37B	3
		T-67O	3
		T-40B	3
		T-36B	3
		T-79O	3
		T-28B	3
		T-78O	3
	2	T-96O	3
		T-90O	3
		T-12G	3
		T-04B	3
	3	T-18G	1
		T-20G	1
		T-21G	1
		T-B092	1
		T-45W*	3
		T-B091	1
		T-32W*	3
	4	T-70O*	1
		T-75O	3
	5	T-08B	3
		T-39G	3
		T-27G	3

*Indicates individuals placed in the wrong full-sib family based on mitochondiral DNA sequence.