

**POPULATION GENETICS OF PACIFIC RIM NATIONAL PARK
RESERVE AND CLAYOQUOT SOUND GRAY WOLVES (*Canis lupus*)**

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1.1 Background:

The gray wolf (*Canis lupus*) is a large, highly mobile carnivore with one of the most expansive natural ranges of any living terrestrial mammalian species (Mech 1970, Nowak 1979, Paquet and Carbyn 2003). In North America, wolves have historically ranged from east to west coasts and from the high Arctic to central Mexico (Mech 1974). This extensive range is due in part to the wolf's ability to disperse over great distances and heterogeneous landscapes in order to find mates and territories (Mech 1970, Mech and Boitani, 2003). Wolves typically disperse <100 km before establishing territories but dispersal may exceed 1000 km (Mech 1970, Fritts 1983, Boyd et al. 1995), with individual daily movements approaching 70 km (Mech and Boitani 2003, Paquet and Carbyn 2003). Wolves are also obligate carnivores that primarily prey on ungulates; however, because they are flexible and opportunistic predators, they may feed on a wide variety of prey, presumably as a function of their presence and availability. However, wolves adapt to local conditions and function as an ecological specialist within the environment it inhabits (Paquet and Carbyn 2003).

The wolves of the Pacific Rim National Park Reserve (PRNPR) and Clayoquot Sound typify this pattern of adaptability. This unique, spatially complex coastline area on the Westside of Vancouver Island hosts a wolf population that deviates from traditional ungulate foraging. In these areas, wolves replace their main prey, Black-tail deer (*Odocoileus hemionus sitkensis*) with other items, which are largely marine. These include river otters (*Lutra canadensis*), various mustelid and other marine-oriented mammals in addition to marine invertebrates. Studies conducted in the PRNPR/Clayoquot Sound area suggest that this deviation in foraging behaviour is due to clear-cut forestry practices in the upper watersheds that run through the park (Bob Hansen, pers. comm.), resulting in a reduction in forage for deer and ultimately a reduction in deer numbers. Estimated numbers of deer within the area is ~5/km² versus 10-20 deer/km² inland of the park boundary (Bob Hansen, pers. comm.). It can be expected that even on a small spatial scale, genetic differences as a result of prey specialization and ecological discontinuities would occur.

Such ecological differences among areas, as well as isolation by geographic barriers, are processes that result in genetic differentiation among sub-populations (Futuyama 1998).

Ecological and behavioural factors such as diet and natal habitat affinity may influence dispersal decisions, which can result in isolation of groups and reveal patterns of genetic structure (Sacks et al. 2004, Pilot et al. 2006, Carmichael et al. 2007, Musiani et al. 2007). However, dispersal capabilities of wolves are high, and they can cross all types of terrain to adjacent habitats (Forbes and Boyd 1996), allowing gene flow to occur and subsequently reducing genetic differentiation. These aspects of wolf behaviour make wolves a valuable test organism for which to examine population genetic structure in the PRNPR and Clayoquot Sound.

In order to investigate such population parameters, Wasser et al. (1997) proposed a non-invasive method for obtaining genetic information using faecal samples. This method allows tracking of animal movements without causing significant disturbance to wildlife as may occur with radio-telemetry collars and tranquilizing techniques. A basic understanding of the PRNPR/Clayoquot Sound wolves of interest, not only in terms of basic ecological information, but also for conservation planning. Population genetic structure and diversity estimates are important in conservation biology, especially in determining whether populations are genetically isolated from each other and the extent of isolation (Johnson et al. 2001). As a result, the paucity of information about population genetics of wolves in the PRNPR/Clayoquot Sound prompted the need to collect baseline information on genetic variation and population structure using non-invasive molecular techniques.

The objectives of this document are three-fold: 1) To obtain baseline genetic information and population structure of PRNPR and Clayoquot Sound wolves. 2) To provide management recommendations based on the genetic results uncovered. 3) To evaluate the effectiveness of non-invasive sampling methods for obtaining genetic information from elusive animals.

1.2 Methods and Materials:

1.2.1 Study Area:

The study area consisted of the PRNPR and Clayoquot Sound, located on the west coast of Vancouver Island. This 207 km North-South distance stretch of land consists of a narrow band of ecologically diverse coastline and island archipelagos. The PRNPR is comprised of three geographically separated park units: the Long Beach Unit (LBU), famous for its beaches, is also a core protected area within the Clayoquot Biosphere Reserve; the Broken Group Islands Unit (BGI) is an archipelago of over 100 islands and the West Coast Trail Unit (WCT), is known world-wide for its 77 km West Coast hiking trail. Clayoquot Sound (CLA) while located outside of the PRNPR is also located within the Clayoquot Biosphere Reserve. These four areas served as sampling sites for this study (Figure 1.1).

1.2.2 Sample collection:

Wolf faecal samples were collected from the study area between 2002 and 2007 at varying times of year, representing over 300 samples. When a wolf faecal sample was encountered, UTM coordinates with a spatial accuracy of 10 metres was recorded. A portion of each sample was preserved in a 50-ml Falcon tube filled with 95% ethanol. Faecal and sampling location characteristics were recorded for each sample, along with any identifiable dietary item present. Three hundred eighteen (318) faecal samples were considered for DNA extraction and genotyping.

1.2.3 DNA extraction and microsatellite amplification:

I performed an optimization procedure to successfully extract faecal DNA as per Navid (2009). DNA extractions were performed in a room physically separated from where amplified PCR products were stored and handled to reduce the risk of contamination. Following optimization procedures, faecal samples were extracted using the QIAamp® DNA Stool Mini Kits produced by Qiagen and the method “Protocol for isolation of DNA from larger amounts of stool” (Qiagen 2007, p.30). Final purified extracts were kept refrigerated at +4°C until use. In total, 200 samples were genotyped.

Microsatellites are a preferred molecular marker used in population genetic studies as they are highly variable, bi-parently inherited, co-dominant and appear to be selectively neutral (Kohn and Wayne 1997). Fourteen microsatellite markers derived from the dog (*C. familiaris* or *C. lupus familiaris*) genome were selected to generate genotypes. These include FH2001, FH2010, FH2017, FH2054, FH2088, FH2096, FH2422 (Breen et al. 2001), FH3313, FH3725 (Guyon et al. 2003), PEZ06, PEZ08, PEZ15, PEZ19 (Halverson J. in Neff et al. 1999), and the Y-chromosome marker MS41B (Sundquist et al. 2001). These markers were chosen for their low number of base pairs ($\sim < 400\text{bp}$) for each allele. Short fragments will amplify better from low quantities of degraded DNA (Frantzen et al. 1998, Murphy et al. 2000). All markers selected except MS41b are tetranucleotide markers, which have been shown to reduce the occurrence of false alleles compared with dinucleotide markers (Taberlet and Luikart 1999). To reduce costs and increase efficiency, markers were arranged into multiplexes, which allow simultaneous amplification of many samples using more than one marker at a time. In comparison with conventional methods, multiplexes have also been shown to reduce genotyping error and increase amplification success (Piggott et al. 2004).

Polymerase Chain Reaction (PCR) conditions optimized for the markers using the Qiagen multiplexing kit were: 95°C/15 minutes (denaturation 94°C/30 seconds, annealing 58°C/90 seconds, extension 72°C/60 seconds) x 35 PCR cycles, final extension 60°C/30 minutes, 15°C/HOLD. The Qiagen multiplexing kit includes a master mix, which contains Taq polymerase enzyme, dNTPs (Deoxyribonucleotide triphosphate), magnesium and buffer, and a Q-solution for augmenting amplification of difficult templates. Protocol for a 10 µl reaction is: 1) Qiagen master mix X2 (5 µl); 2) Q-solution 5X (1 µl); 3) Primer mix 2 µM (1 µl, 0.2 µM final concentration); 4) IRD primer 1 µM (0.4 µl, infrared dye, 0.04 µM final concentration), 5) DNA template (1.5 µl, concentration unknown but likely variable among samples) and 6) sterile H₂O (1.1 µl).

Amplified PCR product was loaded into a 6.5% denaturing polyacrylamide gel, and run on a LICOR4300s DNA analyzer. Genotyping of subsequent fragment alleles was done using LICOR's SAGA GT version 3.3 microsatellite analysis software. I performed DNA extractions at BovaCan Laboratories, at the Saskatchewan Research Council, Saskatoon, SK

and analyses were done by GenServe Laboratories at the Saskatchewan Research Council, Saskatoon, SK.

1.2.4 DNA amplification assessment:

Amplification Rate:

Of the suite of 200 samples submitted for genotyping, I screened and eliminated samples that amplified at less than eight of the 14 loci. This delineation was used as most of the samples are of low DNA quality. By eliminating poor quality samples that may be error prone, I have increased confidence in the genotypes produced, which allows for better matching of genotypes between samples and subsequent analyses (Mowat and Paetkau 2002). I calculated amplification success rate for each locus on the revised number of genotyped samples.

Genetic Identity:

Before any genetic analysis was performed, I used the Excel Microsatellite Toolkit (Park 2001) to check the data set for typographical errors and for samples with identical genotypes. Samples that had alleles matching >75% were considered to be from the same wolf. This delineation was used as it is halfway between the similarity expected between siblings and parent-offspring (50%) and a complete match (100%) (Marco Musiani, pers. comm.).

Error Rates:

As there is generally low quality and quantity of DNA from faecal material when compared with blood and tissue (Talbert and Luikart 1999), higher error rates may occur during genotyping. These include PCR artifacts such as allelic dropout (failure of alleles to amplify, resulting in an over estimation of homozygotes), null alleles (an allele that can no longer be detected due to a mutation at an annealing site, potentially resulting in over-estimation of homozygotes) and generation of false alleles (false positive alleles). Sources of error may obscure true patterns of diversity due to dilution and/or degradation of faecal DNA. Genotyping errors usually produce new individuals because these errors are largely random and the probability of duplication of identical random patterns across multiple loci is

extremely unlikely (Waits and Leberg 2000). This can lead to overestimations in population size.

Although precautions in the laboratory were taken and tetranucleotide microsatellite markers were used, the process of generating genotypes from faecal samples is error prone and quantification of error is necessary. I used two tests to assess error rate. First, to quantify error, 25 samples were re-tested twice and compared them with the original genotypes to provide an error estimate. The re-tested samples were examined to determine if the errors generated were random or if they were specific to certain sampling sites. I built consensus genotypes on this suite of 25 re-tested samples. Second, MICRO-CHECKER 2.2.3 (van Oosterhout et al. 2004) was used to assess possibilities of null alleles, large allele dropout (failure to amplify longer base pair alleles) and scoring errors due to stutter peaks (amplification of an allele immediately before its true amplification peak). Error can also occur due to the presence of other canid species in the area. Because domestic dogs are frequently present in the study area, their faecal samples may have been mistaken as wolf faecal samples.

1.2.5 Standard genetic testing on all loci:

I calculated allelic diversity and observed and expected heterozygosities were calculated for each locus using GENEPOP 3.4 (Raymond and Rousset 1995). Expected heterozygosities were calculated with correction for sample size bias following Nei (1978). The Y chromosome marker MS41b was excluded from genetic diversity analyses to avoid bias in heterozygosity measures between males and females. Fixation indices of heterozygosity (F_{IS}) were calculated per locus according to Weir and Cockerham (1984). F_{IS} is the partition of genetic differentiation between subpopulations due to inbreeding within clusters and measures departures of observed and expected heterozygosity under assumptions of random mating (DeVolo et al. 2005).

I tested genotype gametic disequilibrium (non-random associations of alleles at different loci) and departures from Hardy-Weinberg equilibrium (ratio of expected vs. observed heterozygote genotypes) per locus across all samples in GENEPOP 3.4 using the Markov chain method (Guo and Thompson 1992). I used parameter values from Weckworth

et al. (2005) with global test dememorization number = 10 000, number of batches = 5000, and number of iterations of batches = 10 000. P-values for Hardy-Weinberg equilibrium and gametic disequilibrium tests were adjusted to account for multiple comparisons using the false discovery rate (FDR) as outlined in Verhoeven et al. (2005). This method attempts to correct for both Type I and Type II errors and is preferred over the strict Bonferroni correction, which only corrects for Type I errors.

I examined spatial autocorrelation across all loci using GenAlEx (Genetic Analyses in Excel) version 6 (Peakall and Smouse 2006) with a test of 999 permutations and 1000 bootstrap replicates. Spatial autocorrelation is a more powerful test for assessing fine scale genetic structure than the traditional Mantel tests (Mantel 1967, Peakall et al. 2003, 2006).

1.2.6 Genetic structure:

An exploratory descriptive approach was used to first assess genotypic distributions of individuals. Factorial Correspondence Analysis (FCA) uses multi-locus genetic profiles to plot individuals in a 3-D space, without *a priori* assignments. FCA tries to explain variability in seemingly random variables by using each allele as an independent variable (Roques et al. 2001). I entered all sampling locations (n=4) as potential putative genetic clusters. The program GENETIX 4.0 (Belkhir et al. 2000) was used to compute FCA.

I also examined genetic structure of populations using the Bayesian clustering method implemented in GENELAND version 1.0.5 (Guillot et al. 2005). Unlike other individual-based cluster programs, such as STRUCTURE (Pritchard et al. 2000), GENELAND uses spatial location data for all individuals along with genotypic data to infer the best number of population subdivisions and assign individuals to each. The addition of spatially explicit information is important for identifying cryptic patterns of structure, particularly in fragmented landscapes where barriers to movement or areas differing in ecology might not be so obvious (Coulon et al. 2006).

I examined genetic differentiation between inferred clusters using conventional F -statistics which were calculated with GENEPOP. Pair-wise estimates of F_{ST} using theta (Weir and Cockerham 1984) were used to measure genetic distance between clusters. Gene flow measures, or the number of migrants between genetic clusters was calculated as $Nm =$

$(1 - F_{ST})/4F_{ST}$ (Wright 1969). I also examined genetic distance and gene flow between clusters for males and females separately to determine if sex-biased dispersal was occurring.

1.2.7 Genetic differentiation among clusters:

I performed standard population genetic analyses (Hardy-Weinberg equilibrium, F_{IS} , genotypic gametic disequilibrium) again on the inferred genetic clusters for each of the loci.

1.3 Results:

1.3.1 DNA amplification assessment:

Amplification Rate:

Amplification rates of all loci ranged from 41% to 100% (Table 1.1) with an average amplification rate of 77%. Three markers showed very low amplification success (FH2017, PEZ08 and FH3313) and were noted as potentially problematic for further analysis.

Genetic Identity:

In total, I submitted 200 samples for genotyping and 77 samples (38%) were successful in amplifying at least 8 of 14 markers. I built consensus genotypes on the matching samples identified in the Microsoft Excel Toolkit. A consensus genotype involved combining the genotypic profiles of individuals deemed identical into one individual profile. I generated a total of 44 samples across the study area. The number of samples per sampling area is recorded in Table 1.2. Male wolves were identified by the amplification of the Y chromosome marker and 28 males (41%) were identified in total.

Error Rates:

I calculated error rates for each of the 14 loci and listed them in Table 1.1. Overall, there was an 11% average error rate across all loci, and autosomal loci had varying error rates. The results from the MICROCHECKER analysis are also presented in Table 1.1. All loci except three (FH2096, FH2008 and PEZ 19) showed the presence of null alleles. No

large allele dropout was recorded for any of the loci. The presence of stutter peaks occurred in 43% of the loci which may have led to incorrect assignments of homozygotes/heterozygotes and an overestimation to population size (McKelvey and Schwartz 2004, Selkoe and Toonen 2006).

1.3.2 Standard genetic testing on all loci:

Allelic diversity values including allele number and range for all loci are recorded in Table 1.3. The average number of alleles per locus is 7.21. Values for Hardy-Weinberg equilibrium, F_{IS} , gametic disequilibrium and spatial autocorrelation were calculated across all 44 samples and 13 loci. Observed and expected heterozygosities (H_o and H_e), associated p-values and F_{IS} are also shown in Table 1.3. Eleven of the 13 loci showed departures from Hardy-Weinberg equilibrium with observed levels of heterozygosity lower than expected. F_{IS} values were all largely positive except for FH2096. Significant gametic disequilibrium was also present in 24 of 78 loci pairs after FDR correction.

I plotted spatial autocorrelations of all samples and 13 loci in 20 km intervals across the 207 km study area (Figure 1.2). “R,” the kinship coefficient, was positively correlated up to 30 km, then remained slightly positive between 40-60 km. R-values spiked in a positive direction at both 80 km and 180 km distances. The rest of the area remained slightly positive and within the 95% confidence intervals that spatial structuring is not occurring. Overall, there does not appear to be obvious isolation by distance.

Based on DNA amplification and error rates, along with allelic diversities, I determined that three loci out of the 14 were problematic. FH2017, PEZ08 and FH3313 were eliminated from further genetic analysis. By decreasing the number of loci included in a genotype, the number of errors are reduced which increases the probability that a genotype has been correctly identified (Creel et al. 2003).

1.3.3 Genetic structure:

The results of the FCA are shown in Figure 1.3. Approximately 7% variation exists across all samples in the study area. Samples collected from Broken Group Islands (BGI) show distinct clustering, while the one sample from the West Coast Trail (WCT) appears to

fit best with the BGI cluster. The Long Beach Unit (LBU) cluster shows a slightly more scattered genetic profile while the Clayoquot Sound (CLA) cluster appears to be admixed and without clear structure. Given the proximity of these areas (LBU and CLA) admixture may indeed be occurring (see Figure 1.1). However, with the small sample size of CLA (n=10), one immigrant breeder could be having a disproportionately large effect on the placements of this cluster

GENELAND was run three times to determine K in the study area. All three runs used 50 000 Markov chain Monte Carlo (MCMC) iterations, 44 samples, a minimum population size of one and the Dirichlet frequency model. I also deemed that each sample collected was spatially accurate on this scale of study. I varied the number of maximum populations between K=5-8 along with the number of MCMC computations (132 to 300) based on the sample size. All three runs produced a K=3 population estimate (Figure 1.4). Individuals were assigned to three population clusters: BGI, LBU and CLA. The WCT sample was assigned to the BGI cluster. Four CLA individuals and two BGI individuals appeared to fit best in the LBU cluster, while three LBU individuals appeared to fit better in the CLA cluster. One LBU individual also appeared to fit best in the BGI cluster. These eleven individuals probably represent potential migrants/dispersers within the study area. I performed all subsequent genetic analysis using the GENELAND results to support the objective of obtaining baseline population data for wolves in the study area.

I calculated pair-wise F_{ST} and number of immigrants (N_m) for each cluster to determine genetic distance and gene flow respectively (Table 1.4a-c). Results for F_{ST} range from 0.0289 to 0.176 and N_m ranged from 1.17 to 8.43 immigrants. Genetic distances were greatest between BGI and CLA. Genetic distance and gene flow values for males ranged from 0.160 to 0.196 and 1.03 to 1.31 respectively. Genetic distance and gene flow values for females ranged from 0.014 to 0.154 and 1.37 to 17.61 respectively.

1.3.4 Genetic differentiation among clusters:

Standard genetic testing on inferred clusters for mainland and island areas are shown in Tables 1.5. Average number alleles per cluster ranged from 4.18 (BGI) to 6.00 (LBU). Observed and expected heterozygosities (H_o and H_e), associated p-values and F_{IS} for each

area are also shown in Table 1.5. On average, 4 of 11 loci across all clusters showed departures from Hardy-Weinberg equilibrium with observed levels of heterozygosity lower than expected. F_{IS} values were consistent between clusters with only a negative value for locus FH2096 in the BGI and LBU and a negative value for locus FH2088 in the CLA cluster.

Significant gametic disequilibrium was present in 8 of 78 loci pairs (after FDR correction) in the BGI group, 5 of 78 in the LBU group, and 4 of 78 in CLA. Because loci pairs affected by gametic disequilibrium varied between each cluster and <2 pairs differed significantly between clusters, I retained all eleven loci for analysis.

I ran MICROCHECKER again on each of the inferred genetic clusters. However, the three clusters did not have enough data for MICROCHECKER analysis due to the small sample sizes of each cluster.

1.4 Discussion:

1.4.1 DNA amplification assessment:

Overall, 11 of the 14 microsatellite markers amplified successfully with generally consistent error rates. Error rate was calculated on a subset of retests representative of the study area and appeared to be random and not specific to certain sampling sites. The three markers that amplified poorly (FH2017, PEZ08, FH3313) were excluded.

Individual identification through matching samples required me to balance the risk of combining several similar, but possibly incorrectly genotyped samples as one individual against having multiples of the same individual. The total number of identified individuals across the study area (44) may be relatively high, however, this data set contained samples from 2003 through 2005 and represents both adults and pups from two years.

MICROCHECKER results across all samples revealed the presence of null alleles in all but three markers. Van Oosterhout et al. (2004) notes that null alleles are reported when there is an overall excess of homozygotes distributed evenly across classes, which occurred with my data. The program also warns that if all loci show an excess of homozygotes, the population might not be panmictic (structure occurring as a result of random mating rather

selective mating) thereby making it difficult to separate null alleles from inbreeding and non-panmixia. The absence of large allele drop-out is consistent with choosing markers with a small number of base pairs (Frantzen et al. 1998, Murphy et al. 2000). However, given that tetranucleotide markers were used (except MS41b), I still encountered a 43% stutter peak rate across all loci. Some markers showed a 2 base pair difference between alleles instead of the expected 4, but this could be due to the amplification nature of the locus (Bruce Mann, pers. comm.).

1.4.2 Standard genetic testing on all loci:

Allelic diversity across all loci was comparatively high (7.21) and is consistent with diversities found in other North American wolf populations (e.g. Roy et al. 1994, Forbes and Boyd 1997, Urton 2004, Carmichael 2006, Thiessen 2007, Navid 2009, Stronen 2009). Although comparisons of diversity between different wolf studies using different markers are difficult, the results appear consistent with findings from other areas. Departures from Hardy-Weinberg and gametic equilibrium were significant and may be explained by a few factors. Presence of null alleles, non-random mating and the Wahlund effect could contribute to these deviations (Lehman et al. 1992, Lucchini et al. 2004, Pilot et al. 2006). The Wahlund effect occurs when genetic measures are calculated from one large population when in fact the population is composed of distinct subpopulations, and have been reported in similar studies (e.g.: Pilot et al. 2006, Thiessen 2007, Navid 2009, Stronen 2009). Physical linkage of markers indicated that only two loci (FH2017 and FH2088) were mapped to the same chromosome (CFA15) (Breen et al. 2001), indicating that the gametic disequilibrium is not due to physical linkage. The positive F_{IS} values per locus suggest the presence of inbreeding in all but one locus, but this may be a result of non-random sampling and presence of closely related individuals (Thiessen 2007).

Spatial autocorrelation across all loci does not appear to be significant as kinship values (R) remained mostly within the 95% confidence intervals, suggesting that structuring is not occurring as a result of isolation by distance. Initial positive kinship up to 20 km or larger is expected as it represents allele sharing in familial wolf groups. The positive kinship spike at both 80 km and 180 km suggest that spatial structuring is occurring. This could be

the result of an artifact or potential immigrant(s) as only four alleles and two alleles were associated with each of these deviations respectively. Additionally, given that the study area is relatively small and geographically complex, one may expect some fluctuations in (R) which likely represent geographic features, such as open ocean passages, where wolves cannot cross.

1.4.3 Genetic structure:

The FCA results illustrate the diversity of individual genotypes across the area. Overall, genetic clustering appears to be occurring, particularly in the BGI cluster. The relatively random distribution of CLA could be explained by a few factors. CLA is a small cluster (n=10) and located at the periphery of the study area. It is possible that given the small cluster size, one successful immigrant from either inside or outside the study area could be having a disproportionate effect on the assignment of this cluster. Additionally, the outlier samples could represent the genetic profile of a domestic dog.

The results from GENELAND suggest that most individuals were assigned to three clusters, which are likely different social groups. The BGI cluster appears to be more genetically isolated than any of the other clusters. High mixing is occurring between CLA and LBU clusters, suggesting that effective dispersal is taking place between these areas. LBU and BGI also have moderate gene flow, however the CLA and BGI clusters have extremely limited gene flow and a large genetic distance. The Nm values across the study area combined with the re-assignments of individuals suggest that relatively high gene flow is occurring between at least two of the clusters. The results from GENELAND may also suggest that only two clusters exist in the study area (Figure 1.4), with LBU and CLA comprising one cluster and BGI assigned to the other cluster. This cluster assignment may also be correct as GENELAND is able to detect larger-scale population structure despite the existence of fine-scale familial structure. Sex-biased dispersal, as indicated by Nm values, suggest that females are indeed the dispersing sex and subsequent breeders, particularly between the CLA and LBU clusters (Table 1.4c). Although males are typically the dispersing sex in most mammalian species, female wolves have been documented as dispersing further than male wolves (Kojola et al. 2006).

1.4.4 Genetic differentiation among clusters:

Many population assignment tests assume Hardy Weinberg and gametic equilibrium in inferred clusters. Given the social structure and territorial family groups of wolves, this may be hard to fulfill and violations of these assumptions are expected. However, standard tests of genetic diversity generally support the population assignment of three clusters. Tests of Hardy Weinberg equilibrium, expected heterozygosities and measures of inbreeding also suggest that genetic variation is high and a relatively high level of allelic diversity is maintained within putative clusters. Although random errors in non-invasive data would be expected to scramble genetic structure, my study area shows distinct structuring patterns. Analyses by cluster reduced the number of loci in gametic disequilibrium along with loci not in Hardy-Weinberg equilibrium. These results support that genetic structuring is occurring between clusters in the study area.

I also compared allelic diversities with both BC Central Coast and Riding Mountain National Park (RMNP) wolves (Navid 2009, Stronen 2009) as both areas use the same suite of microsatellite markers. Significant allele diversities and allele ranges were noted for each marker (Table 1.6) when compared to the RMNP wolves. Allele diversities between PRNPR/Clayoquot Sound wolves and BC Central Coast wolves were similar. This may reflect not only the smaller geographic difference between the two areas, but the similarity in foraging patterns. Wolves on the Central Coast of BC also include marine prey in their diets, with island wolves incorporating over 50% marine items (Darimont et al. 2004). The similarity in foraging ecology of PRNPR/Clayoquot wolves and BC Central Coast island wolves may be the reason why similar allele diversities are observed between these two separate wolf populations.

1.4.5 Overall assessment:

The high degree of gene flow between wolf clusters in PRNPR/Clayoquot Sound, particularly in the CLA and LBU sampling sites, imply that wolves are successfully dispersing and reproducing outside of their assigned clusters. Effective dispersal between these adjacent areas may depend on both individual decision making and local population dynamics (Kramer-Schadt et al. 2004). The maximum 207 km linear distance across the

study area at its widest point is easily within recorded dispersal distances of wolves and almost within maximum daily movements (Mech 1970, Mech and Boitani 2003), but the topography of the area suggests that terrain and stretches of ocean, particularly between the LBU archipelago and BGI, might be responsible for the genetic structuring of clusters (Figure 1.5). Because the role of connectivity is critical to the distribution, abundance and persistence of animal populations (Gilpin and Hanski 1991), maintaining connectivity on both land and water will allow for dispersal, gene flow and subsequent maintenance of genetic diversity.

However, it is important to consider explanations other than fragmentation on population structure. Specifically, individuals might be more likely to disperse to and subsequently survive in an area that offers similar prey species, hunting opportunities and challenges. Dispersing grey wolves select habitats similar to the one in which they were reared, and natal habitat-biased dispersal could be influencing gene flow between these areas (Sacks et al. 2004). Thus, the wolf genetic structure uncovered in the PRNPR/Clayoquot Sound may be influenced by any or all of these environmental and ecological factors.

1.6 Management Recommendations:

Based on the genetic structure results outlined above, I make two management recommendations regarding the wolves in PRNPR/Clayoquot Sound and the use of non-invasive methods to obtain genetic information.

1) Continue monitoring all four wolf clusters/sampling sites in the PRNPR/Clayoquot Biosphere Reserve

The results obtained from this initial study meet the objective of obtaining baseline genetic information for PRNPR/Clayoquot sound wolves. However, understanding how gene flow occurs in this study area requires additional information and ecological monitoring. I recommend faecal sampling of all four study area sites to continue for a minimum of one additional year. I also recommend that additional samples should be collected from the WCT study site and that WCT samples already collected should be genotyped. This information will help discern the true number of wolf clusters present in the PRNPR/Clayoquot Sound

study area along with helping to identify potential migrants. The addition of another sampling year will also provide monitoring/observational data which can be compared with results obtained from genetic data. These observational data may also be useful in determining if barriers to wolf movement exist within and between clusters.

Lastly, based on the number of samples collected and number of successful genotypes produced (see Section 1.3.1), I recommend implementing a three-times rule for sample collection. Each study area site should have three-times the amount of wolf faecal samples collected based on the number of individuals the researcher believes exists in each site. This will allow for an adequate number of samples to build consensus genotypes and to fully assess genetic structure.

2) Evaluate the use of non-invasive sampling methods to obtain genetic data from PRNPR/Clayoquot Sound wolves

In the past decade, non-invasive sampling has been employed to obtain genetic information from a variety of species in order to assess demography, gene flow and population structure (see Broquet et al. 2007 for a review). Non-invasive sampling has also been used to infer population structure of elusive, rare and/or endangered species for which invasive methods cannot be used due to ethical concerns of harming individuals (Piggott and Taylor 2003). This study used non-invasive molecular methods to obtain genetic data from elusive gray wolves in the topographically complex coastal band of the Pacific Rim National Park Reserve and Clayoquot Biosphere Reserve.

The key benefit of non-invasive sampling is the reduction of disturbance to wildlife populations (Wasser et al. 1997), which in turn permits population inventories to be conducted on species where physical capture is difficult or undesirable (Paetkau 2003). Additionally, non-invasive sampling methods allow for a greater number of individuals to be sampled over a wider area compared to live capture, radio-telemetry or harvesting approaches (Taberlet and Luikart 1999). However, non-invasively collected genetic samples have inherent limitations. DNA extracts obtained from non-invasive samples are often characterized by low DNA concentration, low DNA quality and/or contamination from alien DNA or laboratory handling (Broquet et al. 2007). Any of these conditions can lead to

genotyping errors such as non-amplification, the generation of false alleles and allelic dropout, which can lead to an overestimation of population size (Taberlet et al. 1996, Taberlet and Luikart, 1999, Creel et al. 2003).

The problems of genotyping error in low quality samples are well understood and various methods have been proposed to limit genotyping errors and their impacts on the subsequent analysis (Bonin et al. 2004, Piggott et al. 2004). By minimizing contamination, following established optimization protocols, amplifying loci using multiplexes, reducing the number of loci used in analysis and effectively assessing error, I was able to decrease genotyping errors and generate baseline population information for PRNPR/Clayoquot wolf clusters without inducing harm on the wolf population. Non-invasive studies cannot be error-free; however, different methods can be used to quantify error and aid in correctly identifying individuals. Probability of identity statistics (P_{ID}) is a theoretical estimator used to quantify genetic diversity levels in populations based on the probability that two individuals drawn at random from a population will have the same genotype at multiple loci (Paetkau and Strobeck 1994, Waits et al. 2001). Additionally, recent capture-mark-recapture models developed for microsatellites may be useful in addressing genotyping errors and providing accurate population estimates (McKelvey and Schwartz 2004, Lukacs and Burnham 2005, Lukacs et al. 2007). Both of these methods may be useful for assessing genotyping errors in wolf faecal samples from the PRNPR/Clayoquot Sound.

1.7 References:

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Table 1.1: Calculations of amplification and error rates and MICROCHECKER results.

*Error rate calculated on a subset of 50 re-tested samples. **Amplification rate calculated on

Locus	Amplification Rate**	Error Rate*	Null Alleles?	Large Allele Dropout?	Stutter Peaks?
	%	%			

all samples that amplified ≥ 8 of 14 loci.

FH2054	93	32	YES	NO	YES
FH2001	98	36	YES	NO	NO
FH2096	100	24	NO	NO	NO
FH2010	89	5	YES	NO	YES
FH2017	50	0	YES	NO	YES
PEZ08	62	1	YES	NO	YES
MS41b	41	1	YES	NO	NO
FH2088	89	8	NO	NO	NO
FH2422	89	9	YES	NO	YES
FH3313	52	4	YES	NO	YES
PEZ06	93	14	YES	NO	NO
PEZ19	77	1	NO	NO	NO
PEZ 15	77	9	YES	NO	NO
FH3725	72	10	YES	NO	NO
MEAN	77	11			

Table 1.2: Number of samples collected from various coastal wolf sites and submitted for genetic analysis (n=44).

Sample Location	Abbreviation	Number of Samples
Broken Group Islands	BGI	18
Long Beach Unit	LBU	15
Clayoquot Sound	CLA	10
West Coast Trail	WCT	1

Table 1.3: Genetic diversity measures of all coastal wolf samples across 14 microsatellite loci. Bold indicates significant p-values. *H_e values are calculated with correction for uneven

Locus	# Alleles	Allelic Range	H _o	H _e *	P-value +S.E.	F _{IS} W&C
FH2054	11	118-170	0.3415	0.8208	0.0000 + (0.0000)	0.5870
FH2001	9	128-154	0.5814	0.7819	0.0005 + (0.0005)	0.2587
FH2096	5	79-103	0.5682	0.4765	0.2876 + (0.0146)	-0.1951
FH2010	4	224-236	0.1282	0.3953	0.0000 + (0.0000)	0.6785
FH2017	3	264-272	0.1364	0.5507	0.0001 + (0.0001)	0.7568
PEZ08	4	197-225	0.0333	0.4164	0.0000 + (0.0000)	0.9212
MS41b	7	205-221	-	-	-	
FH2088	7	72-132	0.6154	0.7479	0.0032 + (0.0013)	0.1791
FH2422	5	182-198	0.2564	0.5914	0.0000 + (0.0000)	0.5696
FH3313	5	337-393	0.1304	0.7024	0.0000 + (0.0000)	0.8177
PEZ06	13	164-196	0.4878	0.8678	0.0000 + (0.0000)	0.4410
PEZ19	5	186-202	0.3824	0.5496	0.0674 + (0.0068)	0.3075
PEZ 15	9	196-260	0.4118	0.7498	0.0000 + (0.0000)	0.4545
FH3725	14	122-194	0.1250	0.8864	0.0000 + (0.0000)	0.8609
MEAN	7.21		0.6567	0.3229	0.0276 + 0.0018	0.5106

samples sizes (Nei 1978).

Table 1.4(a): Pair-wise estimates of F_{ST} genetic distances (above diagonal) and number of migrants (N_m , below diagonal) for all cluster pairs. Bold values indicate smallest and largest genetic distance values.

	Broken Group Islands	Clayoquot Sound	Long Beach Unit
Broken Group Islands N=18	-----	0.1762	0.094
Clayoquot Sound N=9	1.169	-----	0.0288
Long Beach Unit N=17	2.410	8.431	-----

Table 1.4(b): Pair-wise estimates of Male F_{ST} genetic distances (above diagonal) and number of migrants (N_m , below diagonal) for all cluster pairs. Bold values indicate smallest and largest genetic distance values.

MALES	Broken Group Islands	Clayoquot Sound	Long Beach Unit
Broken Group Islands N=10	-----	0.1955	0.1603
Clayoquot Sound N=4	1.029	-----	0.1613
Long Beach Unit N=4	1.310	1.300	-----

Table 1.4(c): Pair-wise estimates of Female F_{ST} genetic distances (above diagonal) and number of migrants (Nm , below diagonal) for all cluster pairs. Bold values indicate smallest and largest genetic distance values.

FEMALES	Broken Group Islands	Clayoquot Sound	Long Beach Unit
Broken Group Islands N=8	-----	0.1539	0.0919
Clayoquot Sound N=5	1.374	-----	0.0140
Long Beach Unit N=13	2.470	17.610	-----

Table 1.5: Genetic diversity measures of each cluster (n=3) based on 10 microsatellite loci. Expected heterozygosity values are calculated with correction for sample size bias. Bold indicates significant p-values.

LOCUS	Broken Group Islands N= 18, allelic diversity = 4.18				Long Beach Unit N=17, allelic diversity =6.00				Clayoquot Sound N=9, allelic diversity = 4.27			
	Ho	He	p-value	F _{IS}	Ho	He	p-value	F _{IS}	Ho	He	p-value	F _{IS}
FH2054	0.4375	9.3548	0.1331	0.2580	0.1875	13.4839	0.0000	0.7830	0.4444	7.1765	0.0319	0.4580
FH2001	0.4706	11.7879	0.0510	0.3280	0.6471	13.9091	0.4149	0.2140	0.6667	7.3529	0.0651	0.1930
FH2096	0.6111	8.1429	0.3193	-0.3650	0.7647	9.6061	0.1426	-0.3680	0.1111	2.6471	0.1737	0.6360
FH2010	0.1250	6.7097	0.0016	0.7090	0.1875	3.7097	0.1912	0.1960	0.0000	3.6923	0.0109	1.0000
FH2088	0.5882	11.5455	0.0928	0.1370	0.5625	12.5484	0.1590	0.2890	0.8333	4.3636	0.3646	-0.1630
FH2422	0.0000	5.3333	0.0001	1.0000	0.4000	11.1034	0.0261	0.4680	0.5714	4.6154	0.8071	0.1430
PEZ06	0.5294	12.0909	0.0221	0.2620	0.5625	14.4194	0.0000	0.3840	0.2500	6.6667	0.0001	0.7140
PEZ19	0.4118	8.8788	0.4161	0.2170	0.3846	7.2400	0.2556	0.3180	0.2500	2.4286	0.1472	0.6250
PEZ 15	0.3333	6.1034	0.1247	0.1860	0.5000	11.1111	0.0127	0.3790	0.4000	3.7778	0.0442	0.5000
FH3725	0.1818	7.9048	0.0000	0.7560	0.0667	12.7241	0.0000	0.9240	0.1667	4.4545	0.0020	0.7920
MEAN	0.36887	8.7852	0.11608	0.3488	0.42631	10.98552	0.12021	0.3587	0.36936	4.71754	0.16468	0.4898

Table 1.6: Comparison of allelic diversity and range between BC Central Coast wolf clusters and Riding Mountain National Park-Duck Mountain wolf populations.

Marker	Pacific Rim National Park Reserve		BC Central Coast		Riding Mountain National Park and Duck Mountain	
	Allelic Diversity	Allelic Range	Allelic Diversity	Allelic Range	Allelic Diversity	Allelic Range
FH2054	10	118-170	9	118-174	12	134-174
FH2001	9	128-154	10	126-154	9	127-152
FH2096	5	79-103	4	95-107	3	95-103
FH2010	4	224-236	6	224-262	5	220-236
FH2017	3	264-272	4	256-272	4	260-272
PEZ08	4	197-225	11	209-257	13	213-253
MS41b	6	205-219	6	209-221	6	211-223
FH2088	7	72-132	5	72-124	7	92-132
FH2422	5	182-198	9	174-210	14	174-242
FH3313	5	337-393	16	305-405	22	337-425
PEZ06	13	164-196	9	174-194	14	164-198
PEZ19	5	186-202	4	194-202	10	182-214
PEZ15	9	196-260	14	200-284	22	204-284
FH3725	13	122-194	7	122-194	20	130-194

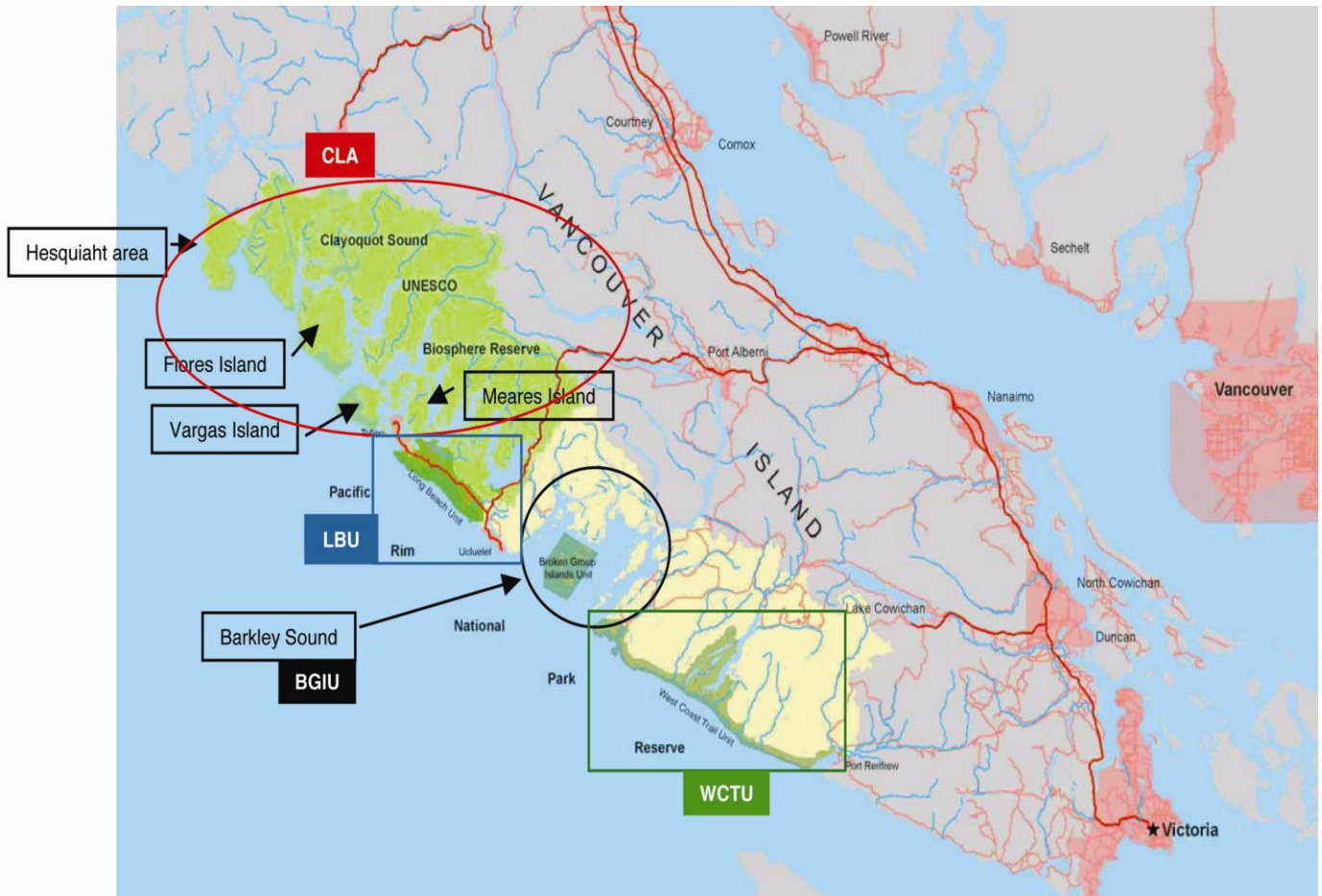


Figure 3.1: Map of study area and sampling sites in Pacific Rim National Park Reserve and Clayoquot Sound (*Source: Bob Hansen*).

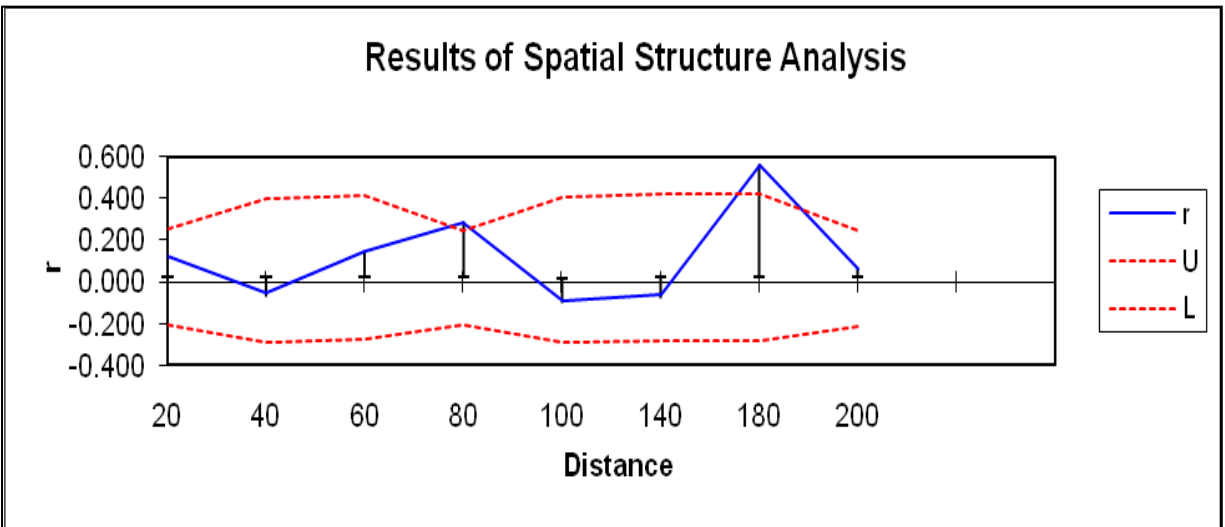


Figure 1.2: Spatial autocorrelation results across all samples and 13 loci. R is the kinship coefficient and is plotted against distance (km). U and L are the upper and lower limits respectively for the 95% confidence interval that identify r values over which no spatial structuring is occurring, as determined by 999 permutations. Error bands show the 95% confidence interval about r, determined by 1000 bootstrap replicates.

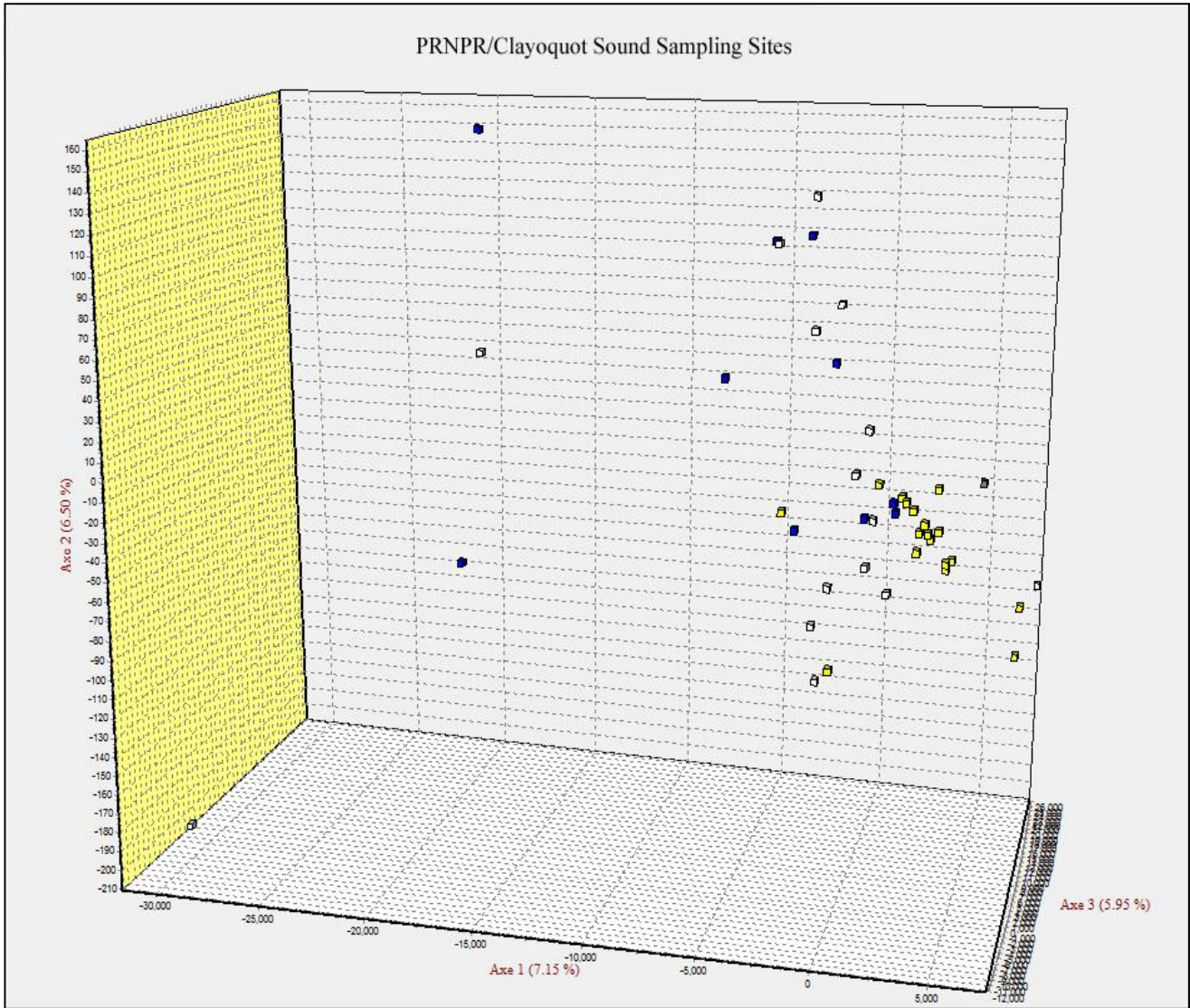


Figure 1.3: Factorial Correspondence Analysis (FCA) of allele distributions of four sampling sites in Pacific Rim National Park Reserve and Clayoquot Sound. Blue: Clayoquot Sound (CLA), Yellow: Broken Group Islands (BGI), White: Long Beach Unit (LBU) Gray: West Coast Trail (WCT). Axis 1 shows most of the genetic variation across the study area.

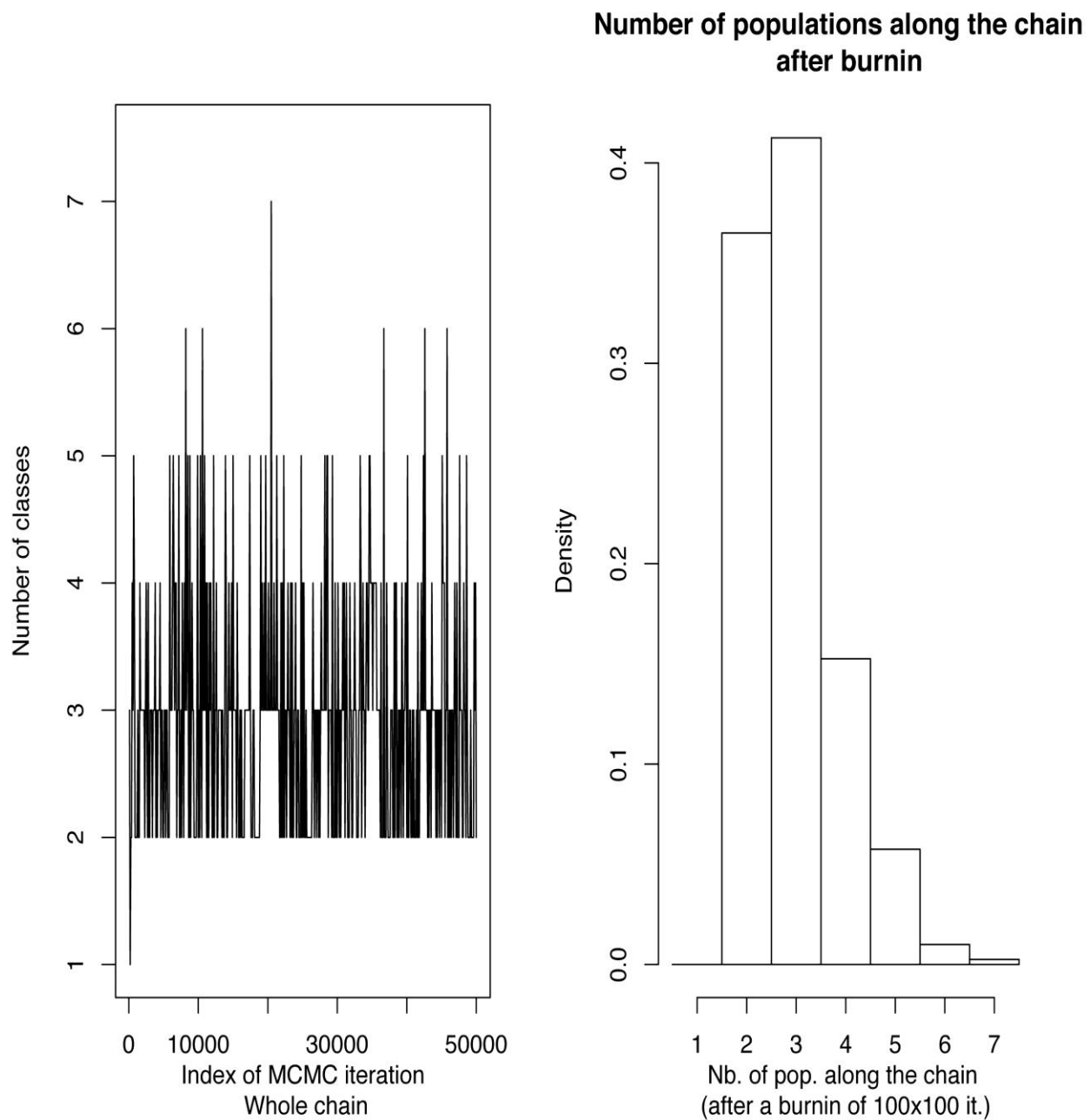


Figure 3.4: Histogram of both the Markov chain Monte Carlo (MCMC) and the number of population clusters as determined by GENELAND.

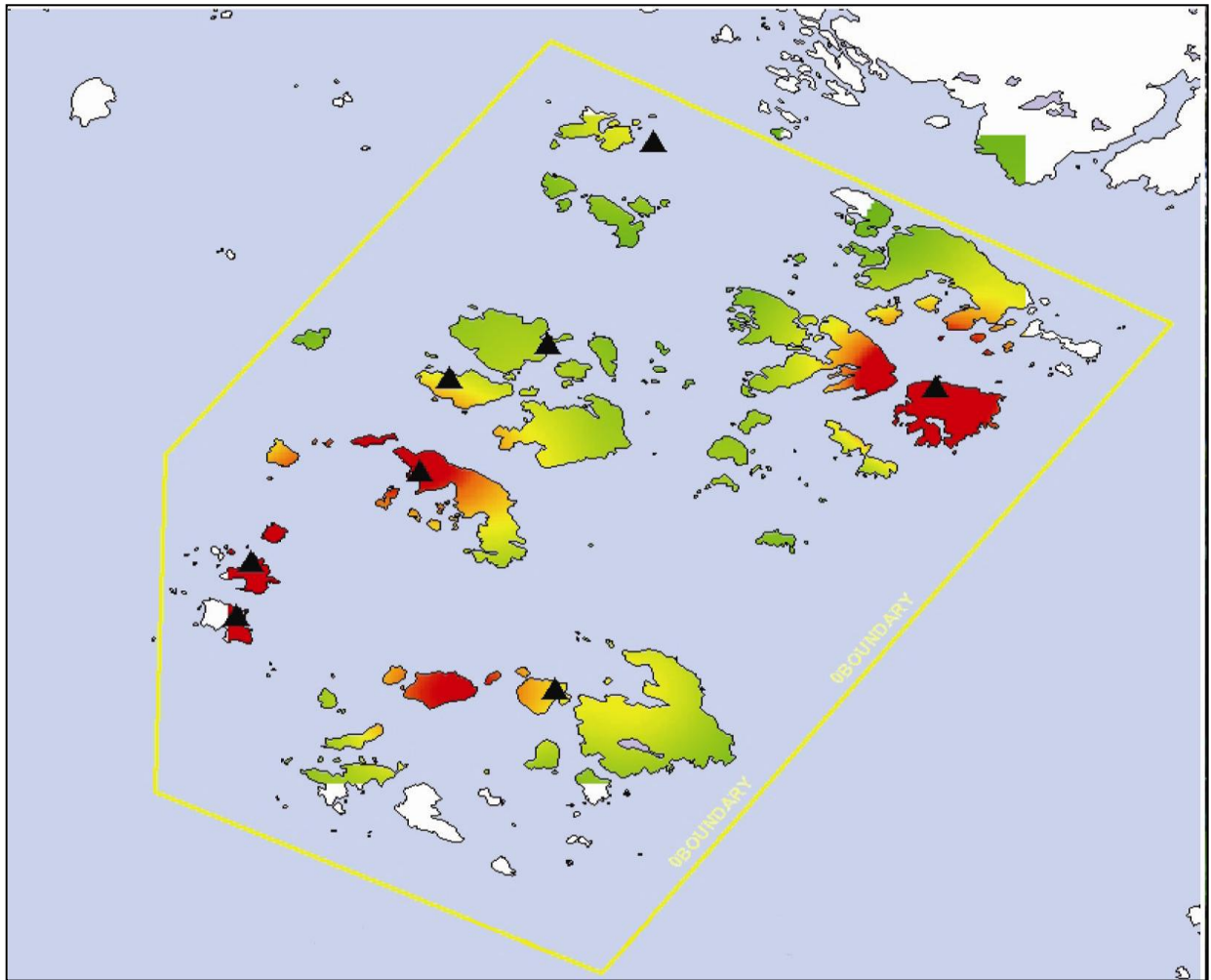


Figure 1.5: Map of Long Beach Unit Island Archipelagos (*Source: Bob Hansen*).