STUDY PLAN
February 5, 2002

Title: Population Genetics of the Pacific Walrus (*Odobenus rosmarus divergens*)

Proposed Starting Date: May 2001

Duration: 5 years

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Introduction
Rationale
The importance of genetic studies in the management of wildlife species is widely recognized. Members of a species are seldom distributed homogeneously in space. Geographical structure is usually caused either by environmental patchiness or social behavior. Smaller spatial units, within which individuals can mate, but among which gene flow is restricted, are the basic units where adaptive evolution takes place. Genotypic data are one of several types of data that are useful in evaluating the evolutionary significance of subpopulations within a species range, an important consideration in formulating management policy (Dizon et al. 1992).

Relatively few studies have investigated the population structure of walruses (*Odobenus rosmarus*). Genotypic determination of population structure in the walrus, and in the Pacific walrus (*O. r. divergens*) specifically, should be a high research priority. No genetic studies of the Pacific walrus have utilized microsatellite DNA, which is now commonly used in population genetic investigations. Comprehensive genetic studies using microsatellite and mitochondrial DNA might reveal distinct subpopulations within the Pacific walrus, and aid in identifying the migration patterns of animal groups. Walrus subpopulations may be uniquely adapted to given areas and may respond to harvest or habitat alterations in different ways, and thus warrant special management considerations. In addition, an increased understanding of walrus distributions and movement patterns will help in the planning and interpretation of future population studies, such as aerial surveys.

Results of this research will provide information about current and past population structure and phylogeographic relationships among aggregations and populations of Pacific walrus. Since similar data have been collected from the Atlantic walrus (Andersen et al. 1998), data from this study can also be combined with those data to assess relationships between Pacific and Atlantic walruses (Lisolette Andersen, pers. comm. to SLT). This information will enable managers to better understand current and historical relationships and to assess the impact of anthropogenic and natural events on walruses in the Pacific, and worldwide.

Distribution and migration
The walrus, *Odobenus rosmarus*, is holarctic in distribution and occurs in at least six geographic regions of the Northern Hemisphere: (1) Hudson Bay-Davis Strait region, (2) eastern Greenland, (3) Svalbard and Franz Josef Land, (4) Kara Sea-Novaya Zemlya region, (5) Laptev Sea, and (6) Bering and Chukchi Seas (Fay 1982). Walruses in the Bering and Chukchi Seas are regarded as the Pacific walrus (*O. r. divergens*), and except for the uncertain taxonomic status of the Laptev Sea walrus, walruses in the other regions are regarded as the Atlantic walrus (*O. r. rosmarus*) (Fay 1982) (Fig. 1).

The general seasonal range-wide distribution of Pacific walruses is known primarily from Fay’s (1982) compilation of published and unpublished historical accounts of walrus sightings from land, ship, and aircraft. In general, female and young walruses utilize sea ice year-round for hauling out, and migrate during fall and spring between the Bering and Chukchi Seas with the annual advance and retreat of sea ice. Adult males generally remain in the Bering Sea year-round, utilizing land haul-outs for much of the year when sea ice is unavailable.
Expanding on ideas of Belopolsky (1939), Fay (1982) postulates that the walrus population is spatially separated during the breeding season in winter into three concentrations: a large concentration in southeastern Bering Sea (from Nunivak and the Pribilof Islands to Bristol Bay), a large concentration in north-central Bering Sea (southwest of St. Lawrence Island), and a smaller concentration in the western Bering Sea (Koryak coast and Anadyr Gulf areas of Russia) (Fig. 2). These concentrations will hereafter be referred to as the Bristol Bay, St. Lawrence, and Anadyr Gulf breeding groups, respectively. Animals from each group may have characteristic routes of migration between their respective wintering and summering grounds, but these have yet to be validated.

During the northward migration of female and young walruses from the Bering Sea in spring, animals from the St. Lawrence group are thought to be the first migrants to pass through the Bering Strait and into the Chukchi Sea (April-May) (Fay 1982). Those from the Bristol Bay group are believed to travel northward along the Alaskan coast and through the Bering Strait mainly in June as a second wave of migrants. Animals from the Anadyr group are thought to travel in spring toward the coastal areas of Anadyr Gulf, and may be joined by some animals from the St. Lawrence group. During summer, these animals travel northward and through the western part of the Bering Strait by September. A small group of adult females and young remain within Anadyr Gulf and utilize land sites with adult males along the southern coast of Chukotka (Mimrin et al. 1990).

Female and young walruses from the Bristol Bay and St. Lawrence groups reach the Chukchi Sea in spring and early summer and divide into two main summering groups, one that travels to Point Barrow and eastward towards Mackenzie Bay, and another that travels to the vicinity of Wrangel and Herald Islands (Fay 1982). Those that reach the Chukchi Sea by late summer and early fall from the Anadyr group might remain within the southwestern area of Chukotka. In late fall, all groups in the Chukchi Sea travel south through the Bering Strait to their respective breeding areas with the southward advance of sea ice.

Most adult males remain in the Bering Sea year-round, but a significant number of animals occupy the northern coast of Chukotka during summer, and a small number of animals occupy areas of the Chukchi Sea with adult females and young. The degree of interannual fidelity of adult males to summering areas is largely unknown. Many adult males from the Bristol Bay breeding group may be the same males that utilize coastal haulouts in this region in summer. Satellite tracking data indicates that at least some adult males from the St. Lawrence breeding group move between this area and the Bristol Bay region in summer (Jay, unpubl. data). Adult males occupying coastal haul-outs in Russia during summer may be comprised of animals derived from the St. Lawrence and Anadyr Gulf breeding groups.

The distinctness of these sexually segregated and mixed groups has not been adequately demonstrated and their boundaries may be quite variable from year to year, particularly those groups in offshore areas, because of the interannual variability in the distribution and extent of sea ice. Furthermore, the degree of philopatry within these proposed groups is unknown.
Past genetic studies
Relatively few studies have used genetics to investigate population structuring in walruses. Cronin et al. (1994) compared mitochondrial DNA (mtDNA) between 30 Atlantic walruses sampled from three sites around Greenland, and 57 Pacific walruses sampled from two areas of the Chukchi Sea (eastern and southwestern Chukchi Sea), and found each of the subspecies to be distinctly monophyletic. Furthermore, based on variations in haplotype frequencies among the sampling locations of Atlantic walruses, they suggest the existence of subdivisions within the Atlantic population. They found no evidence of genetic subdivision between the two groups of Pacific walruses; however, they acknowledge that sampling from the breeding areas in winter, and an analysis of nuclear DNA or regions of mtDNA that are more variable, such as the hypervariable portion of the control region, would be required to adequately assess potential genetic subdivisions within the Pacific walrus population.

Scribner et al. (1997) reviewed the status of genetic studies of the walrus and discussed unpublished nuclear minisatellite and mtDNA data. They used mtDNA to investigate differences in haplotype frequencies among 66 walruses harvested in early spring of 1991 from four areas of the Bering Sea (Nunivak Island, St. Lawrence Island, Anadyr Gulf, and Koryak coast,) and found no significant differences among areas. They also compared their mtDNA results with mtDNA results from animals taken in the Chukchi Sea (collected fall of 1987, n = 57, Cronin et al. 1994), and suggested the possibility of structural differences between the summer (Chukchi Sea) and winter (Bering Sea) groups. However, they suggest that such a difference might only reflect sampling from different portions of the population since samples were collected during different seasons and from different regions of the population’s range. Scribner et al. (1997) also analyzed nuclear multilocus minisatellites to further investigate genetic structuring in samples of Pacific walruses from each of the four locations in the Bering Sea region and in samples of Atlantic walruses from the Greenland region (western and eastern Greenland, n = 15). No evidence of geographic structuring within either region was found. They suggest that further population genetic studies are warranted, particularly those utilizing highly polymorphic genetic markers, and will likely contribute substantially to our knowledge of walrus ecology (Scribner et al. 1997).

Microsatellite DNA may reveal previously undiscovered structuring in walrus populations. Recent studies using microsatellite DNA (and mtDNA) revealed genetic differences between groups of Atlantic walruses (Andersen et al. 1998, Andersen and Born 2000). Differences were found between northwestern Greenland walruses and walruses to the east (eastern Greenland, Franz Joseph Land, and Svalbard) (Andersen et al. 1998), and between northwestern Greenland walruses and a western Greenland group (Andersen and Born 2000). The latter study also found evidence of a connection between the northwestern and western Greenland groups through the migration of males, with only restricted female-mediated gene flow. A review of these studies and additional genetic analyses have confirmed previously identified groups with recognition now of four Atlantic walrus subpopulations (Northwest Greenland, West Greenland, East Greenland, and Svalbard-Franz Josef Land; Born et al. 2001). Mitochondrial DNA has revealed a close familial relationship between females that occur in close proximity to one another, such as on the same small ice floe, within both the Atlantic and Pacific subspecies (Scribner et al. 1997, Andersen et al. 2000).
Goal
The goal of this project is to use microsatellite and sequence information from the hypervariable portion of the control region of the walrus mitochondrial DNA to investigate potential population structuring within the geographic range of the Pacific walrus.

Objectives
1. Determine whether there is evidence of population structure within the geographic range of the Pacific walrus.
2. If structure is evident from Objective #1, identify routes of seasonal migration.
3. Investigate maternal lineages between individuals occurring in groups on ice floes and land haul-out sites.
4. Generate new hypotheses on the distribution, migration, and social organization of Pacific walruses.

Methods

Hypotheses
Four general scenarios are possible (but only three seem tenable) regarding the spatial and genetic relationship of groups of walruses during the breeding period (January-March) and non-breeding period (such as in fall) (Fig. 3). In scenario A, spatially distinct groups during the breeding and non-breeding periods are genetically indistinct (i.e. panmixia).

In scenarios B and C, spatially and genetically distinct groups occur during the breeding period (restricted gene flow among groups), and spatially distinct groups during the non-breeding period are either genetically distinct (scenario B), or indistinct (scenario C), depending on the degree of spatial mixing of individuals during the non-breeding period. If scenario B or C occurs, then it should be possible to sample animals along spring and fall migration corridors to determine the level of spatial mixing that occurs among individuals after they depart from their respective breeding and non-breeding groups, and thus elucidate migration patterns.

In scenario D, behavioral pairing of mates in spatially distinct groups derived from interannual fidelity of individuals to non-breeding areas, form spatially and genetically distinct groups during the non-breeding period. The groups during the breeding period are formed from a mixture of animals from the groups of the non-breeding period. This scenario is unlikely for Pacific walruses, because lekking has been observed in walruses and the sexes are primarily segregated during the non-breeding period.

Evidence will be sought to evaluate one of these four scenarios, for both adult males and females separately, by sampling individuals from spatially distinct groups during the breeding and non-breeding periods. Breeding occurs from January to March. The Bristol Bay, St. Lawrence Island, and Anadyr Gulf breeding groups proposed in Fay (1982) and Mimrin et al. (1990) will comprise the spatial groups of the breeding period to be tested for genetic dissimilarity. These groups are likely associated with the Sireniki, St. Lawrence Island, and Nunivak Island polynyas, respectively (location and extent of polynyas, see Stringer and Groves 1991). Polynyas are predictable areas of the ocean that remain partially or totally ice free, and in the Chukchi and Bering Seas, are caused by winds, currents, or both, and are often on the leeward side of land masses (Smith et al. 1990).
The spatial groups formed in fall (~September) will be tested for genetic dissimilarity (groups identified largely from information in Fay 1982, Mimrin et al. 1990, Rinteimit et al. 2000, and Smirnov et al. 2001). Walruses are maximally segregated by sex, and range at their extreme northern and southern extents during fall, coincident with the maximum northward extent of sea ice (peak about September). For most adult females, these spatial groups are primarily the regions of the eastern Chukchi Sea and the northwestern Chukchi Sea near Wrangel and Herald Islands. Adult females (and large adult males when they occur) will be sampled from these two groups, and from a third and fourth group, the coastal region of northern and southern Chukotka, during fall (September-October, see Fig. 2). For adult males, these non-breeding spatial groups are the regions of Bristol Bay, the coastal region of northern Chukotka, and Anadyr Gulf (including south along the Koryak and Kamchatka coasts).

Allele frequencies of at least 15 microsatellite loci will be used to test for differences among the spatially distinct groups from the breeding and non-breeding periods. Because mitochondrial DNA is maternally inherited and does not undergo recombination, analysis of mtDNA haplotypes will be used to determine maternal lineages and test for female philopatry. The familial structure of small groups of animals on ice floes and beaches will be explored, and gender bias in gene flow will be assessed, by analyzing mitochondrial and nuclear DNA from tissue samples from these individuals.

The following hypotheses will be tested (Objective #1).

(H0: Adult female and male groups during the breeding and non-breeding periods are panmictic.)

**Breeding period**

H1: Adult females from the Bristol Bay, St. Lawrence, and Anadyr Gulf groups during the breeding period (Fig. 2) are genetically distinct.

H2: Adult males from the Bristol Bay, St. Lawrence, and Anadyr Gulf groups during the breeding period are genetically distinct.

**Non-breeding period**

H3: Adult females from the eastern Chukchi, western Chukchi, northern Chukotka coast, and Anadyr Gulf areas during fall (~September) (Fig. 2) are genetically distinct.

H4: Adult males from the Bristol Bay and Anadyr Gulf groups during fall (~September) are genetically distinct.

Contingent upon evidence of genetic structuring from the preceeding hypotheses, (1) additional testing will be done to determine the similarity between breeding and non-breeding groups, and (2) migration patterns will be elucidated by sampling animals at locations along proposed migration corridors during spring and fall and classifying each sample into the breeding and non-breeding group that it is most similar.

**Sample Collection**
Size, location, and timing of sample collection are given in Table 1. It may be prudent to begin sampling along spring and fall migration corridors as opportunities arise, but delay lab analysis
of these samples until results from Objective #1 are known. Many samples for this study can be collected from harvested animals in cooperation with the USFWS Walrus Harvest Monitoring Program (WHMP) and Alaska Native hunters.

Additional samples will be collected opportunistically from live animals. Efforts will be made to sample throughout the range of spatially distinct groups (Fig. 2). To ensure that representative samples are obtained from offshore groups, sampling should be conducted from an icebreaker ship. Future opportunities to work aboard icebreakers in these regions are uncertain. Where possible, samples will be taken at haul-outs or nearshore of suspected offshore groups during the prescribed sampling periods (Table 1) to serve as first approximations to the group’s genetic composition until opportunities to sample by ship in these offshore regions become available. Tissue samples from live animals will be collected during tagging operations and during directed efforts from free-ranging animals when they are hauled out on land and ice. Samples taken during tagging operations will be collected from the animal by excising a small piece of tissue with a scalpel. Samples taken from free-ranging animals will be collected with a biopsy dart delivered by a specially designed dart rifle (PAXARMS, Timaru, New Zealand) or crossbow (WildVet, Victoria, Australia).

Lab and Data Analysis

The USGS Alaska Science Center Molecular Ecology Laboratory (ASC-MEL) in Anchorage, Alaska will analyze all samples and interpret the results. Muscle and other tissue samples will be preserved in non-refrigerated buffer and returned to the ASC-MEL. Laboratory work will proceed in two phases (Phase I and Phase II). For Phase I, a minimum of 30 samples from adult individuals of each gender will be used to genetically characterize each breeding and non-breeding aggregation. Additional collections, made from points on migratory routes during the spring and fall of 2001 through 2003, will be used in Phase II.

For both phases, DNA will be extracted from muscle and other tissue sources using modifications of standard sans-phenol salting out techniques (Medrano et al. 1990) as described in Talbot and Shields (1996). DNA concentrations will be determined using fluorometry. We will use the polymerase chain reaction (PCR) to obtain microsatellite genotypes as well as product for cycle-sequencing of mtDNA for subsequent analyses.

Phase I: Genetic Characterization of Breeding and Non-breeding Aggregations

A minimum of sixty adult individuals (30 females and 30 males) representing each breeding and non-breeding (September) aggregate will be identified to gender and assigned a genotype at each of a series of at least 15 polymorphic microsatellite loci, and a haplotype based on mtDNA sequences for available portions of the mtDNA control region.

Molecular Sexing. Gender of all sampled individuals will be verified using one of several molecular sexing techniques currently used at the ASC-MEL for mammalian species, including canid and ursid species (B. Pierson, unpublished data). At least one of these techniques, accessing the Zinc Finger-Y/X (ZFY/X), a highly conserved mammalian locus residing on both Y and X chromosomes, and the Testes Determining Factor (TDF), a mammalian Y chromosome-specific locus, allows differentiation between male and female walruses (Berthiaume and Talbot, unpublished data).
**Microsatellite Loci.** Currently, 15 biparentally-inherited microsatellite loci, developed for Atlantic walrus and other mammalian species (Kirkpatrick 1992, Allen et al. 1995, Buchanan et al. 1998, Anderson et al. 1998) are sufficiently reliably amplified and polymorphic to be useful for genetic mixed-stock analysis (Talbot, unpublished data). PCR genotyping protocols for each locus will follow optimized protocols currently used in the ASC-MEL for walrus. PCR products will be visualized using a LICOR 4200LR Automated Sequencer, or a LICOR IR2 DNA Analyzer, and analyzed using GeneImageIR™ and/or SAGA™ software.

Tests for significant deviations from Hardy Weinberg and gametic phase equilibrium will be conducted using procedures in GENEPOP 3.2 (Raymond and Rousset 1995). Standard measures of genetic diversity (number of alleles (A), allelic richness ($\delta_R$), and observed (Ho) and expected (He) heterozygosity) will be obtained using ARLEQUIN (Excoffier et al. 1992), and CONTRIB (Petit et al. 1998). Spatial heterogeneity in gene frequency within and among source populations will be examined using FSTAT 9.1 (Goudet 1994). AMOVA (Excoffier et al. 1992) will be used to assess the distribution of allelic variance at different hierarchical levels.

**Mitochondrial DNA.** Nucleotide sequence information from the hypervariable portion of the walrus mitochondrial genome will be collected using cycle sequencing of PCR-amplified DNA, using primers L15962 (Talbot, unpublished) and H00019 (Talbot and Shields 1996). These primers target and amplify a 533-570 portion of the mtDNA of Pacific and Atlantic walrus (Talbot, unpublished data), including a rapidly mutating region homologous to the hypervariable portion 1 of the human mtDNA (Vigilante 1990; Wakely 1993). PCR conditions, purification procedures and cycle-sequencing protocols follow those currently used in the ASC-MEL for walrus species. Data from cycle-sequencing products will be collected using a LICOR 4200LR Automated Sequencer, and analyzed using ImageAnalysis and AlignIR™ software. Should statistical analyses (Pearce et al. 2000) of breeding populations suggest sufficient geographic variation exists among walruses from aggregate locales for these and any additional loci, we will then proceed with the second phase (Phase II) of the study.

Sequence analysis will be conducted as outlined in Talbot and Shields (1996). Measures of genetic diversity (haplotype diversity (h), nucleon diversity ($\pi$), and number of haplotypes (K)) at the mtDNA will be obtained using DnaSP (Rozas and Rozas 1997). Spatial heterogeneity in haplotype frequencies and hierarchical analysis of molecular variance will be conducted using ARLEQUIN (Excoffier et al. 1992).

**Phase II: Genetic Mixed Stock Analysis**
For Phase II, gender will be determined for individuals collected along migratory routes, using the same methods used in Phase I. An appropriate subset (see Pearce et al. 2000) of the microsatellite markers used in the earlier phase will be collected from tissue samples representing various admixed groups. For the microsatellite markers, this would include a modest number of the multiallelic, independently segregating codominant loci tested during Phase I, each characterized by having a small number of alleles, with each allele in moderate frequency (Pearce et al. 2000). The proportional contribution of breeding or non-breeding aggregations to the admixed migrating aggregations will be assessed using mixed stock analysis based on principles of maximum likelihood (Pella et al. 1996, Topchy and Scribner 2001).
Fidelity to non-breeding aggregations will also be tested as for Phase I populations. Laboratory analyses will be conducted during 2001-2003.

Genetic stock identification (GSI) for admixed groups will be conducted for individuals using MLE 1.0 (Topchy and Scribner 2001), on SPAM 3.0 (Alaska Department of Fish and Game 1997) as outlined in Pearce et al. (2000) for mixtures. Genetic markers for GSI will be selected as outlined in Pearce et al. (2000) for examination of admixed groups, or using GENETICA (Topchy 2001) when performing individual assignment to source populations.

**Anticipated Output**
This study will augment field studies by helping delineate phylogeographic relationships among Pacific walruses, and providing information on the relationship between breeding, non-breeding and migration aggregations. Expected data sets include distribution and frequencies of mtDNA haplotypes and nuclear alleles and genotypes of individuals sampled from among and within breeding and non-breeding groups, with graphic representations of phylogeographic relationships among aggregations. Such baseline information are a necessary first step to determine the feasibility of employing genetic stock identification to determine the proportional contribution of breeding populations to non-breeding or migratory admixed groups. These baseline data can also be used as population baseline information necessary to determine the feasibility of using genetic techniques for individual identification in mark-recapture studies (Woods et al. 1999). Results will be submitted for publication in scientific journals.

**Tentative Schedule**
- Spring-summer 2001: USFWS begin WHMP sample collection.
- Fall 2001: Catalog samples in collection at Alaska Science Center.
- 2002-2003: Continue sample collections and analyses.
- 2004: Draft manuscript of findings.
- 2005: Final manuscript for publication.

**Contributions**
For H1-H4 (see Table 1). Does not include salaries or funding associated with existing programs.

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<td>Russian collections (n = 320) (^3)</td>
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1 USFWS Walrus Harvest Monitoring Program.
2 Number of biopsies needed from live animals contingent on success in obtaining samples from other sources.
3 Largely from Russian-US harvest monitoring program and possible sample archives from Wrangel Island.
4 Rough approximation, total largely dependent on needs for additional sample collection.
Literature Cited

Alaska Department of Fish and Game. 1997. SPAM. Statistics program for analyzing mixtures. Version 1.01. Alaska Department of Fish and Game, Division of Commercial Fishing Management and Development, Anchorage, Alaska, USA.


Table 1. Sampling scheme required to elucidate population boundaries and migration routes of the Pacific walrus ($n$, F = female, M = male).

<table>
<thead>
<tr>
<th>Sample location</th>
<th>Breeding period (Jan.-March)</th>
<th>Sample source</th>
<th>Non-breeding period (September)</th>
<th>Sample source</th>
<th>Migration routes</th>
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<td>Zvyagino 1981</td>
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Fig. 1. Worldwide walrus distribution (described in Fay 1982); 1-4: Atlantic walrus (*Odobenus rosmarus rosmarus*), 5: uncertain population affinity, and 6: Pacific walrus (*Odobenus rosmarus divergens*).
Fig. 2. Hypothesized distributions, and spring (April-July) and fall (October-December) migration patterns, of adult female and male Pacific walruses.
Fig. 3. Four potential scenarios of the spatial and genetic relationship of groups of Pacific walruses during the breeding period in winter (January-March) and the non-breeding period (such as in fall): (A) spatially distinct, but genetically indistinct groups during the breeding and non-breeding periods (null hypothesis), (B) spatially and genetically distinct groups during the breeding and non-breeding periods, (C) spatially and genetically distinct groups during the breeding period, and spatially distinct, but genetically indistinct groups during the non-breeding period, and (D) reverse of scenario (C) (scenario D is not a likely for walruses). For simplicity, only two groups are represented in each time period. Arrows indicate movement of individuals between spatial groups and dashed lines indicate genetically similar groups.