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A Determination of Parentage, Mating System, and Genetic Diversity in a Captive Population of the Straw-Colored Fruit Bat (*Eidolon helvum*)

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DISSERTATION APPROVAL

The abstract and dissertation of Jan Zinck for the Doctor of Philosophy in Environmental Science and Resources: Biology were presented on December 6,1999, and were accepted by the dissertation committee and the doctoral program.

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ABSTRACT

An abstract of the dissertation of Jan Zinck for the Doctor of Philosophy in Environmental Sciences and Resources: Biology presented December 6, 1999.

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Small populations tend to lose genetic variability. The magnitude of this loss is influenced by the number of founding individuals, the genetic diversity of the founders, and the species mating system. Genetic variability is the basis of adaptive evolution, and the loss of genetic variability may have harmful effects on development, growth, and survival. Therefore, a primary management goal for small, captive populations is the retention of genetic variability. Of considerable importance to conservation biology is the determination of parentage, from which mating, genetic, and demographic information can be derived. Microsatellites provide a robust molecular evolutionary tool for the study of parentage and genetic variability in populations. When investigating the genetic structure of small populations, mitochondrial DNA (mtDNA), because of its clonal-maternal inheritance, is unparalleled as a marker of maternal relationship. In this study of 123 captive straw-colored fruit bats (*Eidolon helvum*), relatedness, parentage, change in genetic diversity over time, and the captive mating system were evaluated using microsatellite and mtDNA analysis. This study is unique in that founders and twenty subsequent

overlapping generations were analyzed. It represents that first genetic study of this species. Contrary to expectations for small populations, the captive population of *E. helvum* has not experienced a decrease in heterozygosity or allelic diversity, as measured by microsatellite analysis. Neither direct evidence from microsatellite analysis nor gene-drop simulation analysis suggest that genetic drift has played an important role in this population. The captive population of *Eidolon helvum* is well suited for captivity, and has displayed reproductive strategies that minimize the obstacles associated with small populations. A random mating system, rapid population growth, overlapping generations, and long-term, near-equivalent founder contribution have proven to be highly successful in maintaining genetic diversity and demographic stability in this small population. Thus, the reproductive strategy of the captive population of *E. helvum* serves as an excellent model that can be applied to other small populations. It is certainly possible that from the eleven founders a genetically viable, self-sustaining, captive population of *Eidolon helvum* can be established.

A DETERMINATION OF PARENTAGE, MATING SYSTEM, AND GENETIC DIVERSITY IN A CAPTIVE POPULATION OF THE STRAW-COLORED FRUIT BAT (*EIDOLON HELVUM*)

by

JAN ZINCK

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Dedication

To my husband, Steve, for supporting my choice to attend graduate school to earn my doctorate.

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Chapter 1: Introduction

A major focus in conservation biology is on the change in, and distribution of, genetic variability in small, isolated populations over time (Falconer, 1960; Wright, 1969). A small population that experiences a demographic bottleneck is expected to lose genetic diversity, and the distribution of this genetic diversity is expected to change relative to other populations of the same species (Meffe and Carrvel, 1997). Key factors influencing the loss of genetic variability and its distribution include the isolate size, the genetic diversity of the founding population, and the mating system of the species (Richards and Leberg, 1996). The determination of parentage based on genetic data, therefore, is of considerable importance to basic population and conservation biology because it relates directly to mating behavior, population subdivision, effective population size, and management of small populations (Amos et al., 1993; Houlden et al., 1995; McCracken and Bradbury, 1977; Petri et al., 1997). In captive breeding populations, it is also important to know the relatedness of mating individuals in order to avoid the deleterious effects of inbreeding, to minimize damage caused by random catastrophic events, and to monitor the loss of genetic diversity due to genetic drift (Ballou and Foose, 1996; Lynch, 1988; Ralls et al., 1979).

In this study, molecular techniques were developed to be used in the assessment of the captive population of *Eidolon helvum* (straw-colored fruit bat, Kerr, 1792). Maternity and paternity were determined through genetic analysis, and were used to construct an accurate pedigree for this population. Genetic analysis was used

to determine the mating system, to evaluate founder contributions, and to calculate inbreeding coefficients, mean kinship values, and genetic diversity for this captive population of *E. helvum*. Demographic analysis was performed to determine fecundity, mortality, and census parameters. The genetic and demographic data were used to make population management recommendations to establish a genetically viable, self-sustaining, captive population of *E. helvum*.

This chapter begins with a review of conservation genetics. The aspects of the natural history of *E. helvum* that pertain to conservation genetics are then described. The subsequent section describes the molecular techniques used in this research. This chapter is concluded with an outline of the specific goals and hypotheses investigated in this study.

Conservation Genetics

Molecular biology has provided a means for direct investigation of conservation genetics (Avise, 1996). Through the use of a variety of molecular biological methods designed to track genes with a wide range of evolutionary rates, questions ranging from individual to the phylogenetic scope can be addressed.

An understanding of population substructure, gene flow, mating, and reproductive behavior is important when developing management plans in captive or wild populations (Petri et al, 1997). Furthermore, Ballou and Foose (1996) predicted that conservation programs developed for captive colonies may be used for wild populations as well. Preservation of genetic variability and encouraging demographic

stability are the primary ways of maintaining alleles that are the basis of adaptive evolution, and that are valuable to the long-term survival of the species under various conditions (Ballou and Foose, 1996; Soulé, 1980, Soulé et al. 1986). Despite the fact that captive populations often do not experience the same stresses as wild populations (e.g., high parasite loads, exposure to extreme weather, competition for food, poor diet, untreated wounds and illnesses), they do experience unique stresses associated with being in captivity (e.g., the inability to display natural behaviors). Retention of alleles that may provide the variability needed to survive a change or stress is important in captivity, particularly if the species will potentially be returned to the wild (Allendorf, 1986). Loss of genetic variability in small, captive populations may also have harmful effects on development, growth, and survival (Allendorf, 1986).

Difficulties in maintaining genetic variability in viable captive populations stem from the need to retain heterozygosity and to preserve allelic diversity (Fuerst and Maruyama, 1986). Heterozygosity is often insensitive to the actual number of genotypes at a given locus (Allendorf, 1986). Fuerst and Maruyama (1986) suggested that conservation of genetic variability should be interpreted both in light of allele frequency distributions and of heterozygosity of multiple loci. They stated that ignoring allelic diversity in favor of heterozygosity alone when making conservation decisions will likely produce genetically similar or identical populations. Allelic diversity can be lost both during the initial founding event and subsequently due to genetic drift, while heterozygosity is lost at a much slower rate (Fuerst and Maruyama, 1986). The different impacts of heterozygosity and allelic diversity can be thought of

in a temporal sense, with heterozygosity representing a population's ability to evolve in the immediate future, while allelic diversity offers options for responding to selection over the long term (Allendorf, 1986).

In recent years, the zoological community has begun to place an emphasis on conservation through captive propagation and public education (Ballou and Foose, 1996). In a genetic context, all captive populations are small, which makes preserving genetic variability the primary difficulty in captive propagation (Ballou, 1987). As natural populations decline, and as financial and space limitations persist in zoos, small populations will become the norm rather than the exception, both in the wild and in captivity. Therefore, one of the primary goals of captive propagation plans and Species Survival Plans is to maintain self-sustaining populations. Restricted access to wild-born individuals due to quarantine, the lack of wild populations, and the cost of acquiring wild caught animals underscores the importance of self-sustaining populations (Ryder and Fleischer, 1996). Consequently, it is important to track genetic diversity and ensure demographic stability, so as to negate the need for continued founder input. Genetic analyses can be used to identify parentage, define the mating system, and determine effective population size, and other demographic parameters. All of these factors can then be used to develop a management strategy for maintaining genetic diversity and parity of founder contribution in self-sustaining populations. These populations, in turn, may serve conservation purposes to the extent that they retain genetic and behavioral attributes of populations of the same species in the wild.

Genetic studies are crucial to zoo breeding programs. They identify an individual's genetic contribution to a population, which makes inbreeding avoidance possible; they augment pedigree analysis, and can be used to monitor genetic diversity over time (Ryder and Fleischer, 1996). Thus, in addition to providing suitable husbandry and social environments conducive to reproduction, animal managers in zoos must address the genetic well-being of populations of interbreeding individuals. The recent proliferation of Species Survival Plans has been supported and enhanced by numerous genetic research projects (Ryder and Fleischer, 1996). Many of these genetic studies have been performed in captive populations in order to better understand the effects of small population size on genetic variability and gene diversity (Fleischer et al., 1994; Gilbert et al., 1991; Morin et al., 1992; Templeton et al., 1987; Duffield and Wells, 1991.) Pedigree analysis and accurate, complete pedigree listings in studbooks constructed from the results of these genetic studies are crucial to the creation and implementation of Species Survival Plans and captive breeding programs themselves (Ryder, 1986). Furthermore, behavioral and demographic characteristics such as multiple offspring, limited maternal care, or a promiscuous mating system, make constructing an accurate pedigree difficult without genetic analysis. Genetic techniques, such as microsatellite analysis and mitochondrial DNA (mtDNA) restriction fragment length polymorphism (RFLP) analysis, make parentage exclusion and determination possible. With this information, missing or inaccurate pedigree information can be corrected to ensure the predictive value of the pedigree (Ryder, 1986; Ryder and Fleischer, 1996).

Pedigree information has value beyond calculation of genetic parameters affecting a population. Demographic descriptions can be equally useful, and are better calculated from an accurate pedigree. Demographic factors such as mortality and fecundity are important when investigating the life history of a species and its social system, and when making management decisions (Schwartz et al., 1998).

Not all individuals contribute equally to the maintenance of genetic variation. For example, only individuals that successfully reproduce contribute to the subsistence of genetic variation. The number of individuals in a population that prevail in passing their genes to the next generation is called the genetically effective population size (N_e). The effective number of individuals in that population is often much smaller than the actual number of individuals in the population. The effective population size is also influenced by unequal numbers of males and females, unequal family size among individuals of the same sex, fluctuations in population size, and the mating system of the species (Allendorf, 1986, Ballou and Foose, 1996; Meffe and Carrvel, 1997). Pedigree construction, monitoring of captive breeding programs, and the increased problems associated with small effective numbers are further complicated with species such as the captive population of *E. helvum*, which are managed as cross-institutional populations (Ballou and Foose, 1996). Therefore, using accurate pedigree information to determine the effective number of individuals in a population, and understanding the pressures contributing to a reduced N_e, can decrease the potential for genetic consequences of a lowered effective number.

Management of a population's genetic diversity is also important because inbreeding leads to decreased reproductive fitness and higher juvenile mortality. This has generally been referred to as inbreeding depression. Many studies involving both wild and captive populations have demonstrated the detrimental effects of inbreeding depression (Wright, 1977; Ralls and Ballou, 1979; Ralls et al, 1988; DeBoise et al., 1990). The magnitude of this effect depends upon the mating system of the population and the degree to which the population is naturally outbred. Taxa that naturally exist as large populations may experience a more pronounced inbreeding depression when housed in small captive populations than do taxa that commonly inbreed in the wild (Meffe and Carrvel, 1997). When highly-related individuals mate in small isolated populations, homozygosity generally increases and deleterious recessive alleles are expressed. Genetic variation reduces the frequency with which these recessive alleles are expressed, effectively masking their presence in the population.

Preservation of genetic diversity clearly helps to maximize management options available for a captive population (Ballou and Foose, 1996). Once an accurate pedigree has been established and subsequent demographic analysis is complete, management decisions can be made to minimize inbreeding coefficients and mean kinship, and to maximize founder contributions and genetic variability. The combination of the mating system (which can be deduced from the pedigree combined with use of analytical tools such as F statistics) and the distribution of inbreeding coefficients, makes it possible to determine the genetic importance of specific individuals in the breeding population.

Natural History of *Eidolon helvum*

Bats (Chiroptera) are unique among mammals because of their ability to fly. Although they are the second largest order of mammals (after Rodentia), they have received intensive scientific study in only a few areas. Despite great uniformity in physical structure of these animals, they exhibit considerable differences in behavior and lifestyle. The order Chiroptera includes two suborders, Megachiroptera and Microchiroptera. In addition to differences in size and basic morphology, it is generally held that megachiropterans do not echolocate, whereas microchiropterans do.

The taxonomic and ecological diversity found within the Chiroptera have been sufficient to garner interest in them as laboratory and display animals. Their unique physiological and behavioral adaptations (e.g., heterothermy, flight, and echolocation) offer promise for learning more about a variety of other fields from reproduction to navigation (Wilson, 1988). Megachiropterans in particular are kept by many researchers and are frequently found in zoos.

Eidolon is a monotypic genus of the suborder megachiroptera represented by the species *E. helvum* with three recognized subspecies (Hayman and Hill, 1971): *E. h. helvum* (Kerr, 1792), *E. h. dupreanum* (Schlegel and Pollen, 1867), and *E. h. sabaeum* (Andersen, 1907). *E. helvum* lives in the tropical forests of central Africa and Madagascar. The species migrates latitudinally and occurs throughout most of sub-Saharan Africa, including the islands in the Gulf of Guinea, to about 30°S (Jones,

1972). It is the most widely distributed species of the African fruit bats (Nowak, 1994) (Fig. 1.1). *E. helvum* have even been found at sea, 250 km from the nearest land (Rosevear, 1965).

E. helvum bats are most abundant in areas during the rainy season and when fruits are most plentiful. The species displays restricted seasonal monoestry; each female has one litter per year with one offspring per litter (Happold and Happold, 1990). Females are in close reproductive synchrony, mating in May with implantation in October-November and parturition in February-March. Implantation coincides with the beginning of the dry season, synchronizing parturition with the rainy season. Therefore, peak fruit availability occurs simultaneously with lactation and weaning of the young (Jones, 1972; Happold and Happold, 1990). It has been suggested that their mating system of *E. helvum* is based on promiscuity, but whether there is sperm storage and sperm competition is not clear (Fenton, 1985). There has been no systematic genetic analysis of their mating system in the wild or in captivity.

E. helvum roost in large trees 6-20 meters above the ground in colonies upwards of 250,000 individuals. Within these colonies, clusters of a few to 1000 bats are not uncommon. The weight of the clusters frequently breaks the branches of the roost tree. *E. helvum* are alert during the day, often vocalizing and clambering around the roost. The large numbers of individuals within roosts and their excitability make accurate calculations of colony size difficult. Large colony size also makes *E. helvum* colonies highly visible and vulnerable to human attack. Although the wild populations

are currently considered to be stable, the combined effects of habitat loss and over-hunting make the future stability of *E. helvum* uncertain (Fujita and Tuttle, 1991).

E. helvum pollinate and disperse seeds for at least twenty-five known species of plants that are used by humans as food, fuel, medicine, dye, timber, and other economically important products in Africa (Fujita and Tuttle, 1991, Fujita, 1991). Through pollination of highly dependent flowers and dispersal of seeds into forest gaps and clearings where other forest animals seldom venture, these tropical bats play an essential role in forest ecology (Fujita and Tuttle, 1991).

E. helvum exhibits sexual dimorphism with the pelages of adult males being more colorful than females. Male forearms are an average of 13% longer, and males average weight is 250 g while the average female's is 115 g. In West Africa, *E. helvum* is second in size only to the hammer-headed fruit bat, *Hypsignathus monstrosus* (DeFrees and Wilson, 1988).

History of *Eidolon helvum* in American Zoological Association Institutions

In 1979, a mating pair of *E. helvum* was imported from Africa to Seattle's Woodland Park Zoo. An additional seven *E. helvum*, four females and three males, were imported to the Milwaukee County Zoo in 1984. In 1990, the Oregon Zoo imported a breeding pair from Africa. Thus, a total of 11 wild-caught founders for the current captive population of *E. helvum* fruit bats came to exist in American Zoological Association (AZA) institutions. Nine of these eleven founders remained in breeding colonies in 1998, and are included in this study.

The 1998 *Eidolon* studbook (Michel, 1998) reported that the captive population contained 212 bats at 15 institutions. There were 7 breeding colonies, ranging from 6 to 60 individuals each, and 8 non-breeding colonies. Due to the longevity of *E. helvum* in captivity, each subpopulation has a wide range of age classes, from 0 to 20 years of age.

Microsatellite Analysis

Microsatellites are simple-sequence repeats of one to six base-pairs that exhibit high variability in repeat number, which provide alleles that can be unambiguously scored (Queller et al., 1993). Such levels of variability are related to a high mutation rate of 10^{-2} to $5x10^{-6}$ per gamete per locus (Edwards et al., 1992; Hearne et al., 1992). These mutations, resulting in an increase or decrease in the number of repeat units, are thought to be a result of DNA slippage (Schlötterer and Tautz, 1992) and mispairing mechanisms during replication (Schlötterer and Tautz, 1992). Microsatellites are common and regularly dispersed throughout eukaryotic genomes (Tautz, 1989). They are inherited in a co-dominant Mendelian fashion and can be amplified by the polymerase chain reaction, requiring only small amounts of DNA (Tautz, 1989).

Microsatellites provide a robust molecular evolutionary tool for the study of genetic variability in populations (Bruford and Wayne, 1993; Queller et al., 1993) and the application of these genetic markers to population genetics has been utilized for a variety of organisms, including several species of bats (Petri et al., 1997; Queller,1993; Morin et al., 1992; Taylor et al., 1994; Roy et al. 1994; Amos et al.,

1993.). Microsatellites have also been shown to be ideal genetic markers for paternity exclusion and pedigree analysis in both wild and captive populations of mammals (Amos et al., 1993; Craighead et al., 1995; Houlden et al., 1995; Inoue and Takenaka, 1993; Paetkau et al., 1994; Paetkau et al., 1995; Roy et al., 1994; Takenaka et al., 1993; Taylor et al., 1994). Since amplification of microsatellite loci through polymerase chain reaction (PCR) requires only a small amount of DNA, this technique is especially practical for investigating gene diversity, for paternity testing, and ultimately for helping maintain genetic variability in captive and wild populations of animals where sampling is limited (Morin and Woodruff, 1992; Queller et al., 1993; Saiki et al., 1988; Takenaka et al., 1993).

Because microsatellite loci are abundant in eukaryotic genomes and often show length polymorphisms due to differences in the number of tandem repeats, they are ideal for population genetic studies in species that show little or no genetic variation with allozyme analysis or minisatellite fingerprinting (Hughes and Queller, 1993; Houlden et al., 1995; Tautz, 1989; Tautz and Rentz, 1984). Identification of microsatellites is accomplished by the development of primers unique to flanking regions of particular microsatellite sequences. Therefore, this type of DNA analysis provides single-locus genotype information, overcoming the difficulties of multi-locus techniques such as DNA fingerprinting (Weber and May, 1989). For example, with DNA fingerprinting and minisatellite techniques it is not possible to assign paternity without knowledge of the maternal band pattern. Single-locus information provided by microsatellite analysis allows for exclusion of males due to non-matching

genotypes even without knowledge of the maternal alleles (Inoue and Takenaka, 1993).

In captive breeding colonies, microsatellites are used for paternity testing, to investigate genetic variation, and to optimize genetic management of these colonies. Takenaka et al. (1993) used microsatellites to describe aspects of chimpanzee mating behavior, such as a correlation between male rank and number of offspring, female mate selection, and the presence or absence of stable mating pairs. Microsatellites have proved to be especially useful in studies, such as the current investigation, where paternity and mating systems cannot be established reliably by behavioral observation because of the presence of overlapping generations, a multi-male group structure, promiscuity, a large numbers of individuals, or a lack of behavioral data (Amos et al, 1993; Inoue and Takenaka, 1993).

One difficulty with microsatellite analysis is the potential for null alleles. Null alleles are presumed to be mutations in the flanking sequences that affect primer recognition. A null allele mutation may make an offspring appear not to have inherited an allele from one parent, causing heterozygotes to be scored as homozygotes. Heterozygote deficiencies in data sets have been used to alert the investigator to the presence of null alleles (Bruford and Wayne, 1993). For example, using cattle primers to study African buffalo, Simonsen et al. (1998) found that many individual African buffalo scored homozygous at 2 of 6 loci. The resulting heterozygote deficiency prompted Simonsen to develop African buffalo specific primers. Several individuals that were scored as homozygous with cattle primers

scored heterozygous with buffalo primers. The potential for null alleles argues for initially testing the accuracy of primers with known individuals when performing captive studies.

Although microsatellites are generally considered to be selectively neutral, with new mutations showing no bias toward an increase or decrease in repeat number, there has been some evidence of directional selection towards longer repeats. Rubinsztein et al. (1995) compared microsatellite repeat number in humans and non-human primates and found longer repeat motifs in humans. Explanations for this phenomenon include the older age of mating in humans as compared to other non-human primates. Additionally, large populations, such as those of humans, support more neutral genetic diversity and therefore higher levels of heterozygosity than do smaller populations. Therefore, humans may have more conversion events simply because of the size of the population. Rats, with a parallel population increase to humans, also have longer microsatellites (Rubinsztein, et al, 1995).

The possibility of null alleles and directional selection, and a rapid mutation rate, suggest that microsatellite data may need to be evaluated in conjunction with autonomous demographic and population parameters if alleles are to be considered homologous. The current investigation provides an unique opportunity to evaluate population structure through zoological records independent of microsatellite data, thus allowing a calibration of the microsatellite technology for this species.

Mitochondrial DNA Analysis

Mitochondrial DNA (mtDNA) is 16 to 18 kilobases in size, contains genes for 13 proteins, 22 transfer RNAs, 2 ribosomal RNAs, proteins used in electron transport or oxidative phosphorylation, and a regulatory region known as the displacement loop (D-loop) (Wilkinson and Chapman, 1991). Substantial nucleotide sequence and length variation, especially within the D-loop have been reported (Boyce, et al, 1989; La Roche, et al., 1990; Solignac, et al., 1986; Wilkinson and Chapman, 1991). Its small size, ease of isolation, abundance in cells, lack of recombination, and presence of rapidly evolving regions and predominantly maternal inheritance, make mtDNA particularly useful for studying the geographic structure of populations (Hartl and Clark, 1997).

When investigating the genetic structure of small populations, mtDNA, because of its clonal-maternal inheritance, is often more informative than is nuclear DNA, and is unparalleled as a marker of maternal relationships. With an average substitution rate that is approximately five to ten times faster than that in nuclear DNA (Brown, et al., 1979), divergence can be sufficient to distinguish between maternal founders in captive populations. Mitochondrial DNA is, therefore, an excellent tool for elucidating population structure and recent population history (Hartl and Clark, 1997). Mitochondrial DNA analysis by restriction fragment length polymorphism (RFLP) analysis has also been used to evaluate the relationships between populations within species (Cann et al, 1987). Restriction fragment length polymorphisms result from base substitutions or short indels (insertions/deletions) that are detected with the

use of restriction enzymes. Since the inheritance and mode of transmission of mtDNA is well documented for mammalian species, differences in RFLP band patterns can be used to distinguish maternal lines. Although homology of bands that have the same migration pattern must be evaluated, RFLP fragments of identical mobility tend to be homologous in closely related individuals (Dowling et al., 1996). Methylation of DNA can inhibit some restriction enzymes, giving the same result as null alleles in microsatellite analysis. However, this is not a problem when using PCR products as there is no mode for methylation during PCR (Dowling et al., 1996). For pedigree and very recent population structure analysis, RFLP analysis that reveals individual maternal lines offers insight into descendant analysis, founder contribution, and the reproductive success of maternal lineages. Restriction fragment length polymorphism analysis of mtDNA can also be used to investigate mating systems, diversity, parentage, relatedness, geographic variation, hybrid zones, species boundaries, and phylogeny (Dowling et al., 1996).

In captive populations, mtDNA analysis can be used to confirm or assign maternity. For many captive mammals, maternity is assigned by behavioral observation. However, for group-housed, colony, herd, or flock-forming animals, maternity assignment can be confounded. A comparison of maternity assignments made by behavioral observation to those made using molecular techniques can elucidate complex female behaviors, such as stealing of offspring, or alloparenting, which may otherwise go undetected. Mitochondrial DNA analysis has the benefit of

adding to the construction of accurate pedigrees and of providing useful insight into the behavior of the species.

Microsatellite and Mitochondrial DNA Analysis of the American Zoological Association Captive Population of Straw-colored Fruit Bats (*Eidolon helvum*)

The American Zoological Association population of *E. helvum* is an ideal population for microsatellite and mtDNA analysis. The captive population has demonstrated great breeding success, and possesses nearly all the original founding individuals. In addition, zoo records and studbook records are available for the construction of life tables and pedigrees. The breeding success of *E. helvum* in captivity offers an opportunity to investigate the mating system of a species whose large population size in the wild does not facilitate such an investigation. Additionally, the breeding success demonstrated by the captive population of *E. helvum* has created the need to monitor and maintain genetic diversity within colonies over the ensuing generations.

In this study of 123 of the AZA captive *E. helvum*, relatedness, parentage, change in genetic diversity over time, and the captive mating system were evaluated using microsatellite and mtDNA analysis. This study is unique in that founders and 20 subsequent overlapping generations were analyzed, and represents the first genetic study of this species (DeFrees et al., 1988).

Microsatellite primers developed by Dr. Gary McCracken and Lisa Comeaux for bats of the genus Pteropus (University of Tennessee: unpublished, proprietary

information) were utilized. The present investigation also employed mtDNA primers reported by Wilkinson and Chapman (1991) for the evening bat (*Nycticeius humeralis*) to amplify a region of the D-loop between the proline tRNA gene and a conserved region downstream. The presence or absence of an 81 base pair repeat found in the evening bat were evaluated for the *E. helvum*, and random fragment length polymorphism (RFLP) analysis was performed on the amplified mtDNA region.

Goals and Hypotheses

The specific goals of this study were to:

- develop molecular techniques to be used in the assessment of the captive population of *E. helvum*
- assign maternity and paternity to all individuals using exclusion at microsatellite and mtDNA loci
- use maternity and paternity information to construct an accurate pedigree
- use microsatellite genotypes to calculate:
  - expected and observed heterozygosity
  - change in heterozygosity over time
  - change in allele frequencies over time
  - F-statistics
  - Hardy-Weinberg disequilibrium analysis
- analyze the pedigree, genetic, and demographic data to determine the mating system in captive *E. helvum*

- use the pedigree to perform genetic simulations to determine:
  - founder contributions
  - effective number (N_e)
  - inbreeding and mean kinship values
  - founder gene retention
- use the pedigree to perform demographic analysis to determine:
  - fecundity
  - mortality
  - generation time
  - descendant lists
  - reproductive histories
  - census parameters
  - make management recommendations for maintenance of genetic diversity in the captive population of *E. helvum*

It is hypothesized that:

- there will be a decrease in heterozygosity of allelic diversity since founding in the captive population of *E. helvum*
- there will be an increase in inbreeding coefficients and mean kinship over time in the captive population
- the mating system will be characterized by promiscuous mating
- there will be genetic differentiation between subpopulations over time

- there will be unequal founder contributions to the current population

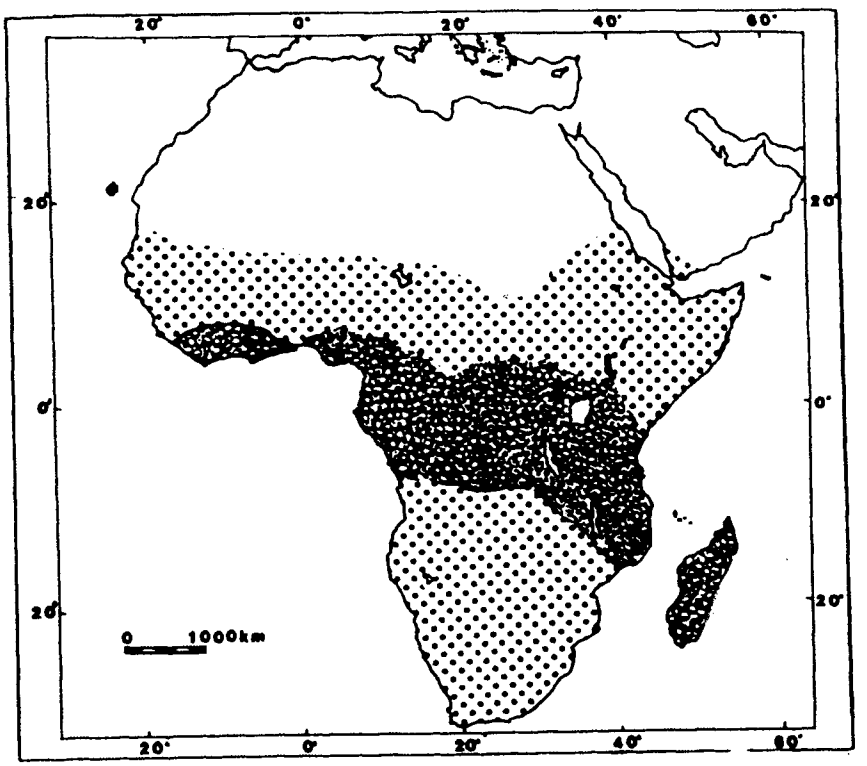

Fig. 1.1 Distribution of *E. helvum*in Africa (adapted from DeFrees and Wilson, 1988). The dark area indicates the central range of *E. helvum*. The shaded area indicates the migratory range of *E. helvum* in Africa.

Chapter 2: Methods

This section describes methods and procedures used for sample collection, DNA extraction and quantification, microsatellite analysis, and mtDNA RFLP analysis.

Sample Collection

Samples were collected between 1996 and 1997 from a total of 123 bats housed at 5 AZA institutions (Table 2.1). Sample collection kits, including one 2 ml screw-cap tube containing 1 ml of silica gel desiccant and one 3 mm-biopsy punch for each bat in the population, were sent to each institution. Tubes were labeled with the animal's identifier and the name of the institution. Veterinarians employed by the zoological institutions, following procedures outlined in Worthington-Wilmer and Barratt (1996), took one 3 mm punch biopsy from each wing of each bat. The two samples were placed in the tube with the silica gel desiccant and were maintained at room temperature, out of direct light, until DNA was extracted.

DNA Extraction

Samples were removed from desiccation tubes and placed into clean 0.5 ml tubes. Each tube then received 450 μL extraction buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM EDTA), 25 μL 20% SDS, and 25 μL 10mg/ml proteinase K. Tubes were

placed in a 55°C water bath for one hour. All tubes were removed from the water bath, gently vortexed, and returned to the water bath overnight.

All tubes were removed from the water bath, and 500 μL of phenol was added to each tube. Tubes were vortexed and then centrifuged for 20 minutes. The aqueous layer of the supernatant was removed and placed in a clean 1.5 ml tube and 500 μL of Chloroform: Iso-amyl alcohol (CIA) (24:1) was added to each tube. Tubes were vortexed and centrifuged for 15 minutes. The aqueous layer of the supernatant was removed and placed in the filter of a Microcon 30 (Amicon). The filter and tube were centrifuged for 10 minutes. One hundred μL of 0.1 X Tris-EDTA (TE) (Sigma) was added to the filter of each tube and the tubes were centrifuged again for 3 minutes. The filters were removed and inverted onto clean 1.5 ml tubes. The filters and tubes were centrifuged for four seconds, five times, resulting in 20-30 μL stock DNA in 0.1 X TE.

DNA Quantification

DNA was quantified using a Hoefer TKO 100 Mini-fluorometer. Working Dye solution (0.1 μg/ml Hoechst dye in 1X TNE (0.1 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 7.4)) was prepared fresh each time DNA was quantified. A 1:10 dilution (100 μg/ml) of 1 mg/ml calf thymus DNA standard solution was prepared by mixing 100 μL DNA with 100 μL 10X TNE and 800 μL H_2O. The glass fluorometry cuvette was filled with 2 ml working dye solution and the fluorometer was adjusted to

zero. Two μL of the dilute (100 μg/ml) DNA standard was added to the cuvette and mixed without introducing bubbles. The fluorometer was adjusted to 100, indicating 100 ng/ml. DNA standard was re-tested until consistent results were achieved. Between samples the cuvette was emptied and new working dye was added to the cuvette. The fluorometer was adjusted to zero between each sample. Two μL of each DNA sample were tested. Readings (ng/ml) were adjusted for the 1/1000 dilution, giving ng/μL of the DNA stock for that individual. Aliquots were taken from the stock and diluted with 0.1 X TE to make a 10 ng/μL solution for use with the polymerase chain reaction (PCR).

Microsatellite Analysis

Microsatellite loci were analyzed using primers developed by McCracken and Comeaux, University of Tennessee (unpublished; Table 2.2). Primers were diluted with ddH_2O to make a 100 μM stock. Aliquots of the stock were diluted with ddH_2O to make a 10 pmole/μL solution for use in PCR amplification.

PCR Amplification

Microsatellite loci were amplified using PCR. When testing for the presence of microsatellite loci, PCR was conducted under non-radioactive ("cold") conditions. Twelve μL reaction volumes were used (see Table 2.3 for optimized conditions). Product was electrophoresed through a 1X Tris-glacial acetic acid-EDTA (TAE) (Sigma), 4% agarose gel and stained with ethidium bromide. Predicted size of the

fragment was confirmed by concurrently running a kilobase ladder (see primer descriptions for predicted size of each microsatellite).

Once PCR conditions were optimized, PCR was conducted under radioactive ("hot") conditions by end-labeling one primer with ^{32}P (Table 2.4). Radioactive PCR reactions for each microsatellite locus are described in Table 2.5. Alleles were separated by electrophoresis through a 20 cmW x 45 cmL 1X TBE, 6% denaturing polyacrylamide/Bis sequencing gel (6% polyacrylamide/Bis, 0.6M Urea) using 0.4 mm spacers. Gels were run at 1100 V with constant voltage for 4.5 hours. Gels were transferred to 3M chromatography paper, covered with a sheet of polyvinyl chloride, and dried using a vacuum gel drier. Fuji PX-B film was exposed to the dried gel overnight at room temperature. The film was developed by submersion in 1X Kodak GBX developer for 2-3 minutes followed by 3 minutes submersion in 1X Kodak GBX Fixer. Alleles were scored using an allelic ladder with an alphanumeric system labeling alleles from largest to smallest.

Mitochondrial DNA RFLP Analysis

Mitochondrial DNA PCR primers F and P, developed by Wilkinson and Chapman (1991), were used to amplify a mtDNA fragment, which was in turn used to narrow the number of possible dams for pups with unknown dams, or to confirm maternity assigned by zoo records. With the reported primers, Wilkinson and Chapman observed length polymorphisms involving an 81 base pair repeat in this region of the mtDNA in vespertilionid bats. However, length polymorphisms in this

region were not observed in *E. helvum*. Because there was no length difference between dams, the five haplotypes corresponding to the original five wild-caught *E. helvum* females were determined by RFLP analysis.

For each mtDNA PCR reaction, 10 pmoles of primer F, 10 pmoles of primer P, and 20 ng template DNA were brought to a total volume of 25 μL with ddH_2O. This mixture was added to a Ready-To-Go PCR Bead tube (Amersham Pharmacia Biotech). Each Ready-To-Go PCR Bead tube, when brought to a final volume of 25 μL, contained 1.5 units of *Taq* DNA polymerase, 10 mM Tris-HCl (pH 9.0 at room temperature), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTP, and bovine serum albumin. Samples were amplified with initial denaturing at 95°C for two minutes. Then 30 cycles of each of the following were performed on each sample: denaturing at 95°C (1 minute), annealing at 53°C (1 minute), and extension at 72°C (1.5 minutes).

Four restriction enzymes for RFLP analysis were used. All restriction digests were comprised of 10μL PCR product, 1μl restriction enzyme (10 U/μL), 1X enzyme buffer, and ddH_2O to 20 μL (Table 2.6). Reaction tubes were incubated at 37°C for one hour. Product was then separated using a 4% agarose E-gel (Invitrogen), visualized using an UV transilluminator, and photographed with Polaroid 667 film. E-gels are bufferless, agarose gels that contain an ion generating system, a pH balancing system, and continuous release of ethidium bromide for DNA staining.

Methods for Statistical Analysis

Microsatellite Analysis

A computer program for the analysis of allelic data, Genetic Data Analysis version 1.0 (GDA) (Lewis and Zaykin, 1999) was used to analyze the microsatellite data. Descriptive statistics, F-statistics, and Hardy-Weinberg disequilibrium were calculated for all loci.

Assignment of Maternity

All maternity assignments or verifications were accomplished manually using genotypic data from this study, and zoo records. For each individual bat, zoo records were used to establish age and presumed maternity. The 1998 Straw-colored Fruit Bat (*Eidolon helvum*) Studbook (Michel, 1998) was used to determine all possible dams for an individual. Maternity was then verified, or determined in ambiguous cases, by exclusion using mtDNA haplotypes and microsatellite genotypes.

Assignment of Paternity

The 1998 Straw-colored Fruit Bat (*Eidolon helvum*) Studbook (Michel, 1998) was used to determine all possible sires for each captive-born bat. Exclusion of possible sires was then based on microsatellite genotypes, and on results from CERVUS (Marshal et al., 1998), a maximum likelihood computer program that determines the most likely sire when one sire cannot be determined by exclusion alone. CERVUS was used to perform three separate sets of analyses: an allele frequency analysis, a simulation to determine confidence limits for assignment of parentage, and a maximum likelihood analysis of candidate sires. The program

ultimately calculated log likelihood ratios (LOD scores) for the possible sires of each mother/pup pair. The LOD scores of the most likely and the next most likely sire were then compared to obtain a Δ value. The calculated Δ value was then compared to the critical Δ value (obtained during the simulation) necessary for each level of confidence (strict, 90%; relaxed, 80%; most likely,< 80%). All sire assignments based on the Δ scores calculated by CERVUS were also evaluated by manual comparison of sire/pup microsatellite genotypes. Thus, the final determination of paternity resulted from direct exclusion by microsatellite data, and from maximum likelihood analysis by CERVUS. For a detailed description of the statistical methods used to create CERVUS see Marshal, et al. (1998).

Pedigree Construction, Demographic, and Genetic Analysis

Maternity and paternity for each individual were recorded into Single Population Analysis and Record Keeping System (SPARKS) software (ISIS, 1991). This software was also used to create the Straw-colored Fruit Bat (*Eidolon helvum*) Studbook (Michel, 1998). Once maternity and paternity data were incorporated into the SPARKS database, demographic and pedigree analyses were performed. Data from SPARKS were then exported to GENES (Lacey, 1990) for the gene drop and founder contribution analysis of the pedigree data.

Table 2-1 Names and Locations of Participating Zoological Institutions

Institution	Location	Number of *E. helvum* sampled
Lincoln Park Zoological Gardens	2200 N Cannon Dr. Chicago, IL 60614	15
Franklin Park Zoo	1 Franklin Park Rd. Boston, MA 02121	6
Milwaukee County Zoological Gardens	10001 W. Bluemound Rd., Milwaukee, WI 53226	60
Oregon Zoo	4001 SW Canyon Rd., Portland, OR 97221	35
Woodland Park Zoological Gardens	5500 Phinney AVE. N, Seattle, WA 98103	7

Table 2-2 Description of Microsatellite Primers

Primer	Repeat	Average length of PCR product
P-9	$(GT)_{12}(AT)_7$	222 base pairs
P-4	$(TTA)_8$	259 base pairs
P-18	$(CTTT)_{32}$	208 base pairs

Table 2-3 Amplification Characteristics for "cold" PCR

Locus	DNA (ng)	Primer (pmoles)[1]	Polymerase (μL)	$MgCl_2$ (mM)	PCR conditions[2]
P-9	20	10	0.2	1	D:95, A:55, E:72 (28N)
P-4	20	10	0.2	1	D:95, A:61, E:72 (28N)
P-18	10	10	0.2	3.5	D:95, A:55, E:72[3] (28N)

Polymerase is Taq Polymerase (5 U/μL) from Promega

[1]Concentration is given for each primer

[2] For P-9 and P-4, PCR was carried out as follows: each tube contained 1.2 μL of 10X reaction buffer (Promega), 0.5 μL of 5 mM dNTPs, and ddH_2O to a total reaction volume of 12 μL. Each PCR protocol began with a 2 minute initial denaturation at 95°C, followed by Denaturation (D), Annealing (A), and Extension (E) at the temperature [°C] indicated, for one minute each temperature, for (N) cycles.

[3] For P-18, PCR was carried out as follows: each tube contained 2.4 μL Buffer D (Invitrogen) (Concentrations in a 12 μL reaction = 60 mM Tris-HCL, 15 mM ammonium sulfate, 3.5 mM $Mg\ Cl_2$, pH 8.5), 1.2 μL 10mM dNTPs, and ddH_20 to a total reaction volume of 12 μL. Each PCR protocol began with a 2 minute initial denaturation at 95°C, followed by Denaturation (D), Annealing (A), and Extension (E) at the temperature [°C] indicated, for one minute each temperature, for (N) cycles.

Table 2-4 End-labeling Reactions of "hot" PCR

Primer (μl)	T4 Buffer (μl)	T4 Kinase (μl)	32P dATP (uCi)	Conditions
0.67	1	0.5	8	Incubate @ 37°C for 30 minutes, boil for 2 minutes

Above conditions represent 10ul reactions and are brought to volume with ddH_20.

Table 2-5 Amplification Characteristics for "hot" PCR

Primer	End-labeled Primer (μL)	unlabeled primer (μL)	DNA (ng)	Polymerase (μL)	$MgCl_2$ (Mm)	PCR Conditions[1]
P-4	1	0.067	20	0.2	1	D:95 (1min.), A:61(30 sec.), E:72 (30 sec.) N26
P-9	1	0.067	20	0.2	1	D:95 (1min.), A:55 (1 min.), E: 72 (1 min.), N26
P-18	1	0.067	20	0.2	3.5	D: 95 (1 min)[2] A: 55 (1.5 min) E: 72 (2 min) N28

[1] PCR was carried out as follows: each tube contained 1.2 μL of 10X reaction buffer (Promega)), 0.5 μL of 5 mM dNTPs, and ddH_2O to a total reaction volume of 12 μL. Each PCR protocol began with a 2 minute initial denaturation at 95°C, followed by Denaturation (D), Annealing (A), and Extension (E) at the temperature [°C] indicated, for (X) cycles.

[2] PCR was carried out as follows: each tube contained 2.4 μL Buffer D (Invitrogen) (Concentrations in a 12 μL reaction = 60 mM Tris-HCL, 15 mM ammonium sulfate, 3.5 mM $Mg\ Cl_2$, pH 8.5), 1.2 μL 10mM dNTPs, and ddH_20 to a total reaction volume of 12 μL. Each PCR protocol began with a 2 minute initial denaturation at 95°C, followed by Denaturation (D), Annealing (A), and Extension (E) at the temperature [°C] indicated, for (N) cycles.

Table 2-6 Description of Restriction Enzymes Used for RFLP Analysis

Enzyme	Recognition site	Buffer
Sau96 1 BioLabs	5'...G↓GNCC...3' 3'...CCNG↑G...5'	NE Buffer 4
Taq 1 (GibcoBRL)	5'...T↓CGA...3' 3'...AGC↑T...5'	REact 2
Hinf 1 (GibcoBRL)	5'...G↓ANTC...3' 3'...CTNA↑G...5'	REact 2
Hpa II (BioLabs)	5'...C↓CGG...3' 3'...GGC↑C...5'	NE Buffer 1

Chapter 3: Results

Microsatellite Alleles

Three variable microsatellite loci called P-4, P-9, and P-18, with 4-7 alleles each, were utilized in this study. An allelic composite showing all alleles defined in this study is given in Fig. 3.1. CERVUS was used to calculate allele frequencies and the number of heterozygous and homozygous individuals for each locus using the microsatellite genotypes of all individuals scored (Table 3.1).

The exclusionary power of a locus or set of loci represents the power of that locus/loci to exclude a randomly selected unrelated candidate parent from the parentage of an arbitrary offspring. Exclusionary power is expressed as the probability of excluding an unrelated candidate parent. In this study, the exclusionary power at individual loci, calculated by CERVUS, ranged from 0.207-0.251 for the first and 0.365-0.409 second parent. The total exclusionary power for these microsatellite loci taken together was 0.55 for the first parent and 0.78 for the second parent (Table 3.2).

Mitochondrial Haplotypes

The four restriction enzymes used in this study resulted in 10 haplotypes. A detailed description of all haplotypes is presented in Table 3.3. Visualization of the restriction fragments is given in Figure 3.2.

Individual Identification

Each individual's genotypic data from microsatellite analysis and each individual's mtDNA haplotype are presented in Appendix A. The individual's local I.D. and studbook number are indicated along with the sex of the individual. Maternity and paternity (if known) are also indicated.

Maternity and Paternity Assignment

Maternity was assigned using zoo records, mtDNA haplotypes, and microsatellite genotypes. Potential dams were excluded using mtDNA haplotypes, with microsatellite loci being used to exclude dams with the same haplotype. In a few cases, mutations at microsatellite and mtDNA loci caused mismatches, which will be discussed later. Maternity was assigned or confirmed for 79.5% of the total offspring in this study. In cases where maternity was assigned in the studbook and where samples were available for this study, maternity assigned by genetic analysis agreed with the studbook designation 79% of the time. Appendix B shows the dam/pup assignments determined using genetic analysis for all captive-born offspring in this study. Each dam's genotype, and the genetic contribution to her offspring, are shown in bold type. Any mismatches between maternal and pup genotypes are indicated with shading. Offspring are listed in order of birth year.

Paternity was determined, as described in Materials and Methods, using zoo records, exclusion, and maximum likelihood calculations (CERVUS). 73% of the total possible sires were sampled. Paternity was assigned for 13.7% of the total

offspring at the 90% confidence limit, 24.5% at the 80% or greater confidence limit, 40.2% at the <80% confidence limit, with the remaining 4.9% of sires determined manually using zoo records and exclusion. Appendix C gives the sire/pup assignments for all captive-born offspring in this study. The confidence level associated with each assignment is indicated with (*) 90%, (+) 80%, and (–) <80%. Individuals without symbols were assigned paternity manually. As in Appendix 4, parental genotypes and paternal genetic contributions are indicated in bold type. Pups are listed in order of birth year. Pups without genotypic data are individuals that were not included in this study and have been assigned by zoo records only.

Of all the captive-born individuals sampled in this study, there remain 20.5% with unknown maternity, 30.4% with unknown paternity, and 5.8% with neither parent known.

Heterozygosity

The number of subjects scored (n), the expected (He) and observed (Ho) heterozygosities, and the estimate of the fixation index (F) for each individual locus were calculated using GDA for each captive colony (Tables 3.4-3.6), and the population as a whole (Table 3.7). Observed heterozygosity for these loci ranged from 0.33 to 1.0. Expected levels of heterozygosity were calculated assuming Hardy-Weinberg equilibrium for the locus/loci in each subpopulation. Thus, any deviation from expected heterozygosity represents departure from Hardy-Weinberg equilibrium.

The fixation index, which quantifies any departure from equilibrium, is a ratio composed of expected and observed heterozygosities.

$$F = \frac{He - Ho}{He}$$

A fixation index of zero represents no deviation from Hardy-Weinberg equilibrium with respect to the number of heterozygotes. A positive fixation index indicates fewer heterozygotes than expected, and a negative fixation index indicates an excess of heterozygotes over expected. Analysis of expected and observed heterozygosities and the fixation indexes (Tables 3.4-3.7) revealed that in most cases the current subpopulations contained the number of heterozygous individuals expected from Hardy-Weinberg equilibrium.

Observation of heterozygosity over time shows that the current heterozygosity has been stable for over 10 years, with most change being attributed to an increase in heterozygosity at locus-P-18 (Fig. 3.3). In the four years between 1984 and 1988, heterozygosities at all loci converged towards 0.6, and have remained relatively stable since that time.

Hardy-Weinberg Disequilibrium

Hardy-Weinberg disequilibrium probabilities sample all possible sets of genotypic frequencies from the observed allele frequencies, and reject the hypothesis of equilibrium if the observed genotypic frequencies are very unusual (Weir, 1996). Hardy-Weinberg disequilibrium probabilities were calculated by GDA for each locus

using allele frequencies within each subpopulation (Table 3.8-3.12). The number of runs indicated in the tables is the number of replicate times that genotypic frequencies were drawn from the observed allele frequencies. The resulting distribution of genotypic frequencies was used to determine the probability of the observed genotypic frequency for each subpopulation under Hardy-Weinberg equilibrium. A probability less than or equal to 0.05 represents significant Hardy-Weinberg disequilibrium. Evaluation of individual subpopulations at individual loci indicated Hardy-Weinberg disequilibrium at locus-4 in Milwaukee ($p = 0.01$) and at locus-P-9 in Portland ($p = 0.002$). All other loci in all other subpopulations were in Hardy-Weinberg equilibrium.

F-Statistics

F-statistics assume random mating and calculate the reduction in heterozygosity expected at one level of a population hierarchy relative to another more inclusive level of the population hierarchy (Hartl and Clark, 1997). Subscripts are used to indicate the level of population hierarchy being evaluated (I = individual, S = subpopulation, T = total population).

F-statistics were calculated using GDA. F_{IS}, F_{IT}, F_{ST} values for each locus, each allele within each locus, and all loci combined, are presented in Table 3.13. F-statistics involving the total population are calculated using the average genotype frequencies across all subpopulations. Although in general each subpopulation is in Hardy-Weinberg equilibrium, as shown previously, these subpopulation may each

have a different allele frequency, and therefore may have different expected heterozygosities. Because there is not random mating between subpopulations, genetic differentiation between subpopulations can be evaluated using F_{ST}. When $F_{ST} = 0$ there has been no genetic differentiation between subpopulations for the locus/loci being evaluated, whereas when $F_{ST} = 1$ there has been fixation of a different allele at the locus/loci in each subpopulation.

Bootstrap analysis was calculated using GDA, where subsets of data were drawn with replacement and the F-statistics were re-calculated each of 5000 times (Table 3.14). Neither F_{IS} nor F_{IT} were statistically different from 0, as indicated by the upper and lower bounds narrowly overlapping zero, while F_{ST} was statistically different from zero, as indicated by the non-overlap of zero by the upper and lower bounds. This suggests that there has been genetic differentiation between subpopulations. The F_{ST} in this study range from 0.003 – 0.03, indicating that the level of genetic differentiation between subpopulations is still quite low (Wright, 1978).

Change In Allele Frequencies Since Founding

Allele frequencies were manually determined using genotypes of individuals identified by zoo records as being founders or as being present in the current population. Comparisons of allele frequencies between founders and the current population, and between subpopulation founders and current subpopulations in Portland and Milwaukee, are shown in Figs. 3.4-3.6. There has been a change in the

frequency of alleles C and D, locus-P-18 (p = 0.005 and 0.05 respectively), and of alleles D and E, locus-P-9 (p = 0.005, 0.05 respectively) in the current population. Allele D must have been present in founder #2 because her offspring, #50, has allele D, locus-P-9 and #50's known sire does not. Male founder #10 may also have had this allele. The significant change in allele E, locus-P-9 can be attributed to male founder #32's lack of reproduction. There has been significant change in the frequency of alleles A and D, locus-P-18 in the Portland subpopulation (p = 0.05, 0.005). Additionally, there was a change in the frequency of allele D, locus-P-9 (p = 0.005). There has been significant change in the frequency of alleles A, C, and D, locus-P-18 in the Milwaukee subpopulation since founding (p = 0.005 (A and C), 0.025 (D)). There was also a change in the frequency of alleles 1 and B, locus-P-9 (0.005 and 0.05 respectively), and allele B, locus-P-4 (p = 0.025).

In a small population, genetic drift would be expected to affect allele frequencies over time. Allele frequencies in the captive population of *E. helvum* each year between 1984 and 1998 are illustrated for each locus in Fig. 3.7 – 3.9. These comparisons illustrate that for most of the loci, the frequencies of alleles have been stable over time between current and founding populations. Most of the changes in allele frequencies that did occur happened within the first five years, and allele frequencies then stabilized. It is notable that there has been no loss of alleles since founding in the captive population of *E. helvum*.

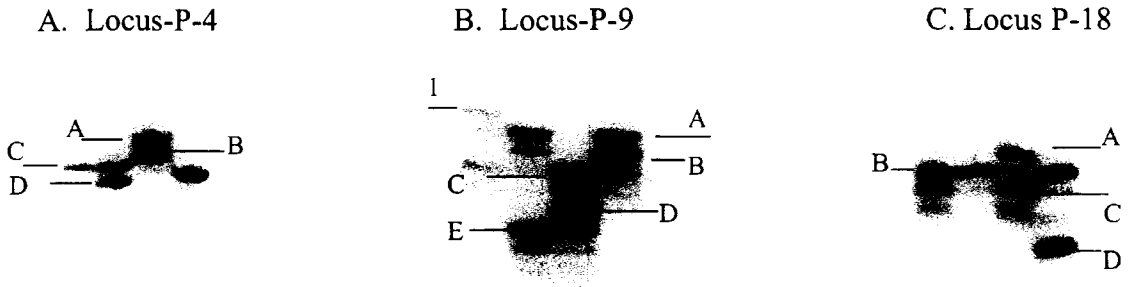

Fig. 3.1 Autoradiographs showing alleles at each microsatellite locus. A. Locus-P-4 has four alleles. Alleles are scored alphabetically from largest to smallest. B. Locus-P-9 has six alleles. The largest allele, 1, was scored numerically because it was not observed in the population until the second year of analysis. All other alleles at this locus are scored alphabetically from largest to smallest. All bands at this locus have one dark primary band and one light stutter band. Alleles were scored using the primary band. C. Locus-P-18 has four alleles. Allele C has one dark primary band and one light stutter band. This allele was scored using the primary band.

Table 3-1 Allele Frequencies and Counts of Homozygotes and Heterozygotes at Each Locus

A. Locus P-4

Allele	Count	Allele Frequency	Heterozygotes	Homozygotes
A	30	0.130	24	3
B	15	0.065	13	1
C	62	0.270	44	9
D	123	0.535	59	32

Counts were the number of individuals that had the indicated allele in the total population. The allele frequency was also calculated for the total population. The numbers of heterozygotes and homozygotes indicate the proportion of individuals from the count that are homozygous or heterozygous for the allele. These calculations were performed using CERVUS.

B. Locus P-18

Allele	Count	Allele Frequency	Heterozygotes	Homozygotes
A	18	0.085	18	0
B	99	0.467	59	20
C	60	0.283	46	7
D	35	0.165	27	4

Counts were the number of individuals that had the indicated allele in the total population. The allele frequency was also calculated for the total population. The numbers of heterozygotes and homozygotes indicate the proportion of individuals from the count that are homozygous or heterozygous for the allele. These calculations were performed using CERVUS.

C. Locus P-9

Allele	Count	Allele Frequency	Heterozygotes	Homozygotes
1	6	0.027	6	0
A	22	0.098	20	1
B	51	0.228	49	1
C	116	0.518	56	30
D	25	0.112	17	4
E	2	0.009	2	0
F	2	0.009	0	1

Counts were the number of individuals that had the indicated allele in the total population. The allele frequency was also calculated for the total population. The numbers of heterozygotes and homozygotes indicate the proportion of individuals from the count that are homozygous or heterozygous for the allele. These calculations were performed using CERVUS.

Table 3-2 Exclusionary Power of Individual and Combined Microsatellite Loci

Locus	Exclusionary Power (first parent)	Exclusionary Power (second parent)
P-4	0.207	0.365
P-9	0.251	0.425
P-18	0.245	0.409
Combined	0.551	0.784

Exclusionary power is the probability of excluding a randomly selected unrelated candidate parent from parentage of an offspring. Exclusionary power is calculated as the probability of excluding a parent when only the genotypes of the offspring and candidate parent are known (first parent), and the probability of excluding a candidate parent when the genotypes of the offspring, a known parent, and the candidate are known (second parent). These calculations were performed using CERVUS.

Table 3-3 Description of Mitochondrial RFLP Haplotypes

Haplotype	Sau96 1	Taq 1	Hinf 1	Hpa II
Type I	Multiple fragments ≅ 300 bp	2 fragments: 200 bp, 170	No restriction	No restriction
Type II	Multiple fragments ≅ 300 bp	2 fragments: 200 bp, 170 bp	No restriction	2 fragments:300bp and 60 bp
Type III	Multiple fragments ≅200 bp	2 fragments: 200 bp, 170 bp	No restriction	N/A
Type IV	Multiple fragments ≅ 300 bp	2 fragments: 200 bp, 130 bp	No restriction	N/A
Type V	Multiple fragments ≅ 300 bp	2 fragments: 200 bp, 170 bp	3 fragments: 100 bp, 200 bp, 60 bp	N/A
Type VI	Multiple fragments ≅ 300 bp	No restriction	2 fragments: 200 bp, 160 bp	N/A
Type VII	Multiple fragments ≅ 300 bp	No restriction	2 fragments: 240bp, 120 bp	N/A
Type VIII	Multiple fragments ≅ 300 bp	2 fragments: 200 bp, 170 bp	3 fragments: 180 bp, 160 bp, 20 bp	N/A
Type IX	Multiple fragments ≅ 300 bp	No restriction	3 fragments: 100 bp, 200 bp, 60 bp	N/A
Type X	Multiple fragments ≅200 bp	2 fragments: 200 bp, 170 bp	2 fragments: 200 bp, 160 bp	N/A

This table indicates the band pattern produced by each of four restriction enzymes used in this study. The band pattern from all enzymes is evaluated to determine the haplotype of an individual. Fig. 3.2 shows the band patterns observed for each restriction enzyme.

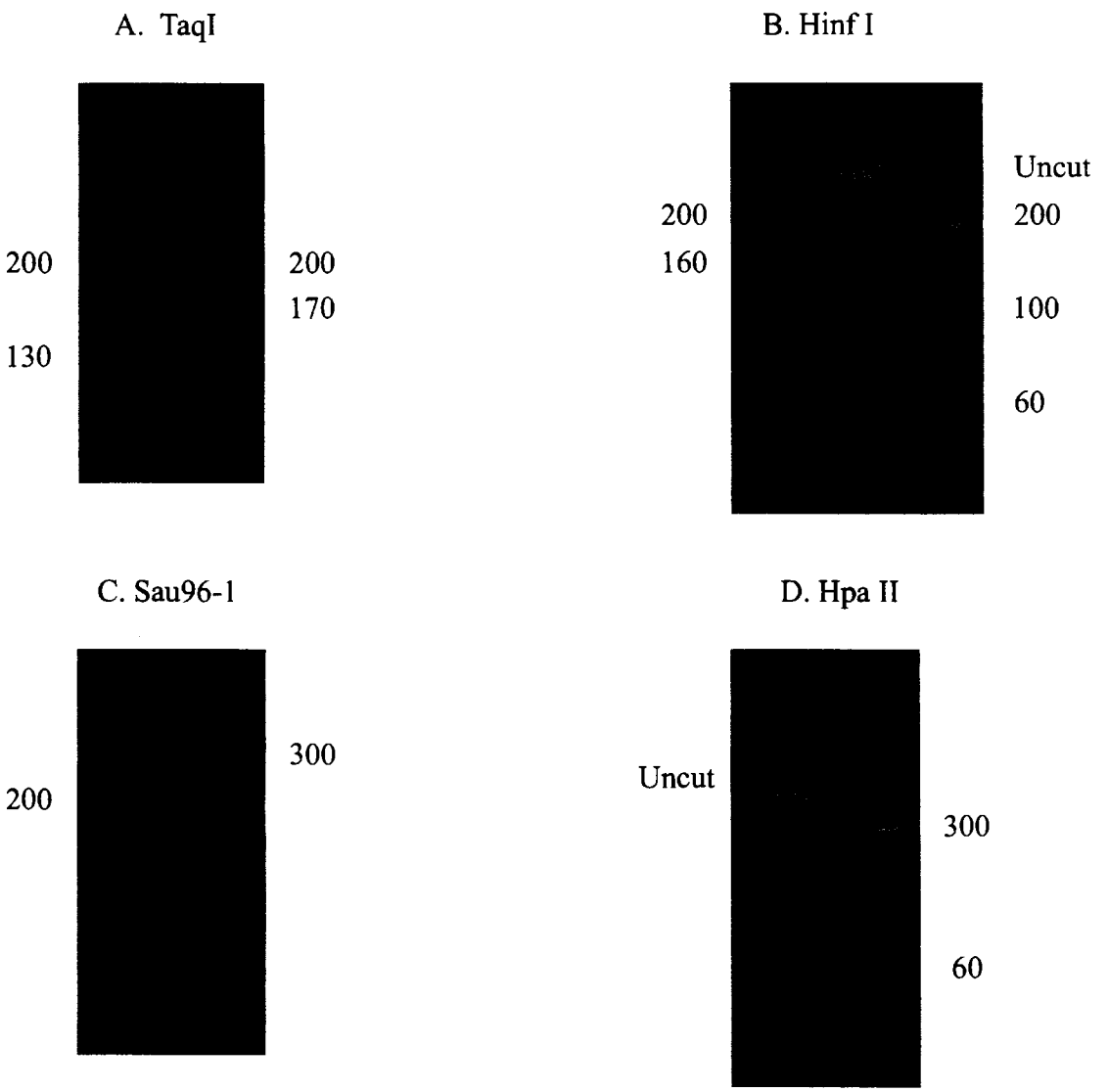

Fig. 3.2 Restriction fragments resulting from the digestion of a PCR amplified section of mtDNA. The approximate size of the restriction fragments are indicated to the left and right of each picture. A. Restriction digests with Taq I resulted in two band patterns. B. Restriction digests with HinfI resulted in either no restriction, or two different band patterns. C. Restriction digests with Sau96-1 resulted in two different band patterns. D. Restriction digests with HpaII resulted in either no restriction, or one cut. The combined results from each restriction digest determined the mitochondrial haplotype of each individual bat. Table 3.2 describes the unique restriction pattern for each of the ten observed mitochondrial haplotypes.

Table 3-4 Descriptive Statistics for Locus-P-4

Subpopulation	n	A	Ho	He	F
Milwaukee	58	4	0.586	0.666	0.121
Portland	30	4	0.600	0.524	-0.149
Franklin Park	6	3	0.333	0.530	0.394
Chicago	15	3	0.600	0.559	-0.077
Seattle	6	2	0.667	0.485	-0.429
Total	115	4			
Mean			0.557	0.553	-0.009

n = sample size, A = number of alleles/loci, He = expected heterozygosity, Ho = observed heterozygosity, F = estimation of fixation index. The fixation index is a ratio of expected to observed heterozygosity, with a value of zero indicating Hardy-Weinberg equilibrium. Positive values for F indicate less heterozygotes than expected and a negative value indicates more heterozygotes than expected. These calculations were performed using GDA 1.0.

Table 3-5 Descriptive Statistics for Locus-P-9

Subpopulation	n	A	He	Ho	F
Milwaukee	53	5	0.628	0.736	-0.174
Portland	31	7	0.645	0.516	0.203
Franklin Park	6	4	0.652	0.667	-0.026
Chicago	15	5	0.618	0.733	-0.194
Seattle	7	5	0.758	0.714	0.063
Total	112	7			
Mean			0.660	0.673	-0.063

n = sample size, A = number of alleles/loci, He = expected heterozygosity, Ho = observed heterozygosity, f = estimation of fixation index. The fixation index is a ratio of expected to observed heterozygosity, with a value of zero indicating Hardy-Weinberg equilibrium. Positive values for F indicate less heterozygotes than expected and a negative value indicates more heterozygotes than expected. These calculations were performed using GDA 1.0.

Table 3-6 Descriptive Statistics for Locus-P-18

Subpopulation	n	A	He	Ho	F
Milwaukee	57	4	0.649	0.737	-0.137
Portland	25	4	0.714	0.600	0.163
Franklin Park	6	3	0.712	1.00	-0.463
Chicago	14	4	0.712	0.786	-0.109
Seattle	6	2	0.530	0.50	0.063
Total	108	4			
Mean			0.663	0.725	-0.094

n = sample size, A = number of alleles/loci, He = expected heterozygosity, Ho = observed heterozygosity, f = estimation of fixation index. The fixation index is a ratio of expected to observed heterozygosity, with a value of zero indicating Hardy-Weinberg equilibrium. Positive values for F indicate less heterozygotes than expected and a negative value indicates more heterozygotes than expected. These calculations were performed using GDA 1.0.

Table 3-7 Descriptive Statistics Over All Loci

Subpopulation	N	He	Ho	F
Milwaukee	56.0	0.648	0.686	-0.060
Portland	28.7	0.628	0.572	0.090
Franklin Park	6.0	0.631	0.667	-0.062
Chicago	14.7	0.630	0.706	-0.127
Seattle	6.3	0.591	0.627	-0.065
Mean	22.3	0.625	0.652	-0.043

n = average sample size, He = expected heterozygosity, Ho = observed heterozygosity, f = estimation of fixation index. The fixation index is a ratio of expected to observed heterozygosity, with a value of zero indicating Hardy-Weinberg equilibrium. Positive values for F indicate less heterozygotes than expected and a negative value indicates more heterozygotes than expected. These calculations were performed using GDA 1.0.

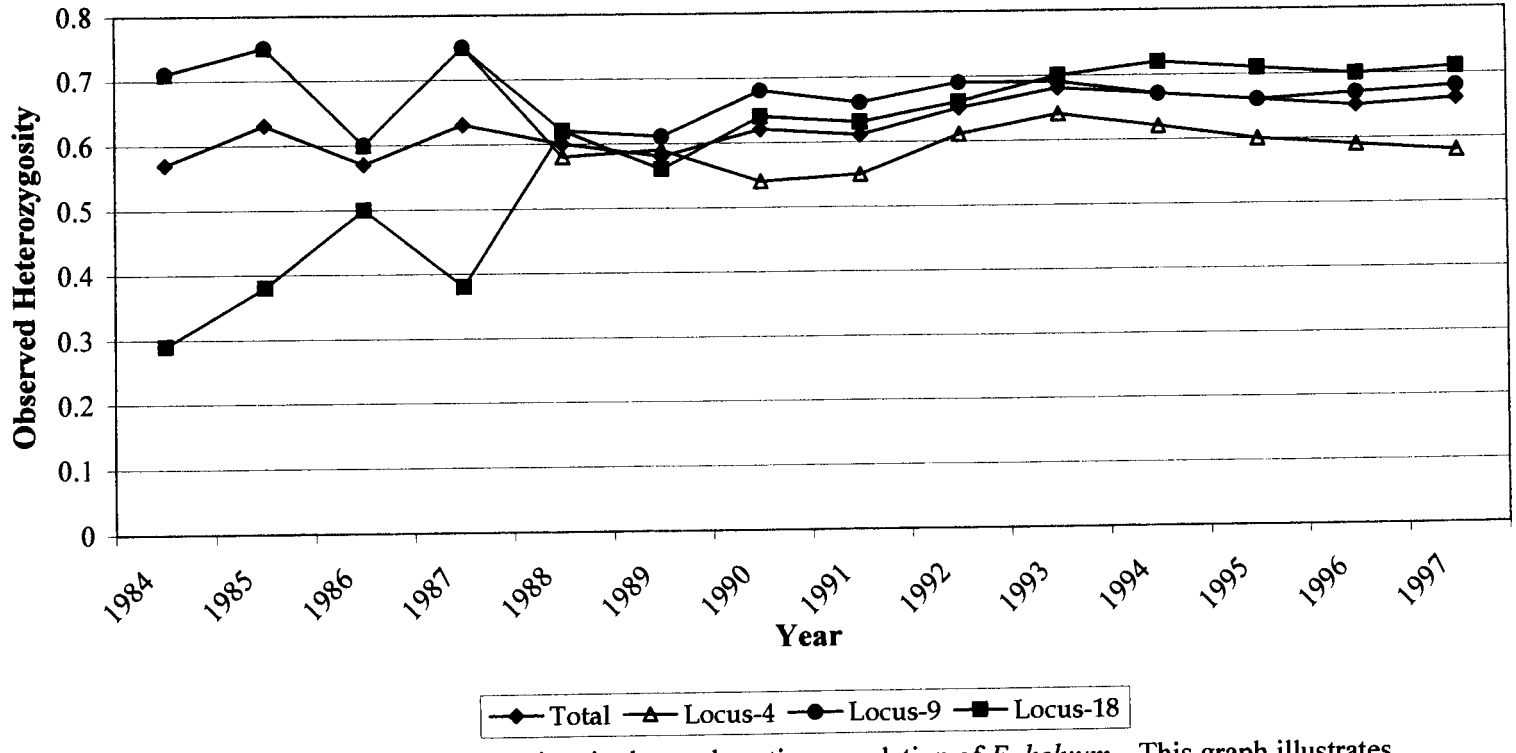

Fig. 3.3 Change in heterozygosity over time in the total captive population of *E. helvum.* This graph illustrates the change in heterozygosity at each locus and at all loci combined each year since founding. Due to overlapping generations, many of the founding and subsequent individuals remain in the current population. Heterozygosity at all loci converged to 0.6 between 1984 and 1988, and have remained relatively stable. There has not been a loss in heterozygosity between the 1984 founding population and the 1998 population. Heterozygosity was calculated using GDA 1.0.

Table 3-8 Exact Tests For Hardy-Weinberg Disequilibrium: Milwaukee, 60 Individuals

Runs	Probability	Locus Combination
3200	0.009687*	Locus-P-4
3200	0.117188	Locus-P-9
3200	0.550625	Locus-P-18

The exact tests for Hardy-Weinberg disequilibrium calculate the probability of the observed genotype frequencies from the observed allele frequencies. A distribution of possible genotype frequencies and the probability of each frequency is created through repeated sampling (Runs) of genotype frequencies from the observed allele frequencies. A probability of less than 0.05 indicates disequilibrium. Locus-P-4 is in disequilibrium in the Milwaukee subpopulation. These calculations were performed using GDA 1.0.

Table 3-9 Exact Tests For Hardy-Weinberg Disequilibrium: Portland, 32 Individuals

Runs	Probability	Locus combination
3200	0.695000	Locus-P-4
3200	0.002500*	Locus-P-9
3200	0.264375	Locus-P-18

The exact tests for Hardy-Weinberg disequilibrium calculate the probability of the observed genotype frequencies from the observed allele frequencies. A distribution of possible genotype frequencies and the probability of each frequency is created through repeated sampling (Runs) of genotype frequencies from the observed allele frequencies. A probability of less than 0.05 indicates disequilibrium. Locus-P-9 is in disequilibrium in the Portland subpopulation. These calculations were performed using GDA 1.0.

Table 3-10 Exact tests For Hardy-Weinberg Disequilibrium: Franklin Park, 6 Individuals

Runs	Probability	Locus combination
3200	0.273750	Locus-P-4
3200	0.509062	Locus-P-9
3200	0.588125	Locus-P-18

The exact tests for Hardy-Weinberg disequilibrium calculate the probability of the observed genotype frequencies from the observed allele frequencies. A distribution of possible genotype frequencies and the probability of each frequency is created through repeated sampling (Runs) of genotype frequencies from the observed allele frequencies. A probability of less than 0.05 indicates disequilibrium. There are no loci in disequilibrium in the Franklin Park subpopulation. These calculations were performed using GDA 1.0.

Table 3-11 Exact Tests For Hardy-Weinberg Disequilibrium: Chicago, 15 Individuals

Runs	Probability	Locus combination
3200	1.00	Locus-P-4
3200	0.2300	Locus-P-9
3200	0.386875	Locus-P-18

The exact tests for Hardy-Weinberg disequilibrium calculate the probability of the observed genotype frequencies from the observed allele frequencies. A distribution of possible genotype frequencies and the probability of each frequency is created through repeated sampling (Runs) of genotype frequencies from the observed allele frequencies. A probability of less than 0.05 indicates disequilibrium. There are no loci in disequilibrium in the Chicago subpopulation. These calculations were performed using GDA 1.0.

Table 3-12 Exact Tests For Hardy-Weinberg Disequilibrium: Seattle, 7 Individuals

Runs	Probability	Locus combination
3200	1.00	Locus-P-4
3200	0.6900	Locus-P-9
3200	1.00	Locus-P-18

The exact tests for Hardy-Weinberg disequilibrium calculate the probability of the observed genotype frequencies from the observed allele frequencies. A distribution of possible genotype frequencies and the probability of each frequency is created through repeated sampling (Runs) of genotype frequencies from the observed allele frequencies. A probability of less than 0.05 indicates disequilibrium. There are no loci in disequilibrium in the Seattle subpopulation. These calculations were performed using GDA 1.0.

Table 3-13 F-Statistics With 3 Loci and 5 Subpopulations

Locus	Allele	F_{IS}	F_{IT}	F_{ST}
P-4	All	0.029	0.061	0.033
	D	-0.026	-0.007	0.018
	C	0.046	0.063	0.018
	B	0.041	0.138	0.102
	A	0.114	0.163	0.055
P-9	All	-0.047	-0.012	0.033
	F	1.000	1.000	-0.019
	E	-0.023	0.004	0.026
	D	0.237	0.240	0.004
	C	-0.022	0.012	0.034
	B	-0.296	-0.267	0.022
	A	-0.083	0.030	0.104
	1	-0.009	-0.021	-0.030
P-18	All	-0.067	0.077	0.017
	D	-0.061	0.077	0.017
	C	-0.059	-0.074	-0.014
	B	-0.118	-0.136	-0.016
	A	-0.152	-0.054	-0.016
Overall		-0.030	-0.007	0.023

F-statistics calculate the reduction in heterozygosity expected at one level of a population hierarchy relative to another more inclusive level of the population hierarchy. The level of the population hierarchy being evaluated is indicated with subscripts (I = individual, S = subpopulation, T = total population). Genetic differentiation between subpopulations would be indicated by a positive F_{ST}. These calculations were performed using GDA 1.0.

Table 3-14 Results From Bootstrap Analysis Over All Loci

Bound	F_{IS}	F_{IT}	F_{ST}
Upper	0.028670	0.060586	0.032987
Lower	-0.066852	-0.063197	0.003426
No. Reps.	5000	5000	5000

Bootstrap analysis of the F-statistics calculated for the captive population of *E. helvum*. Subsets of data were drawn, with replacement, 5000 times (No. Reps.) and the F-statistics were re-calculated. The upper and lower bounds are the highest and lowest values calculated during bootstrap analysis. F_{IS} and F_{IT} narrowly overlap zero, and F_{ST} is positive. This indicates a slight differentiation between subpopulation (Wright, 1978). These calculations were performed using GDA 1.0.

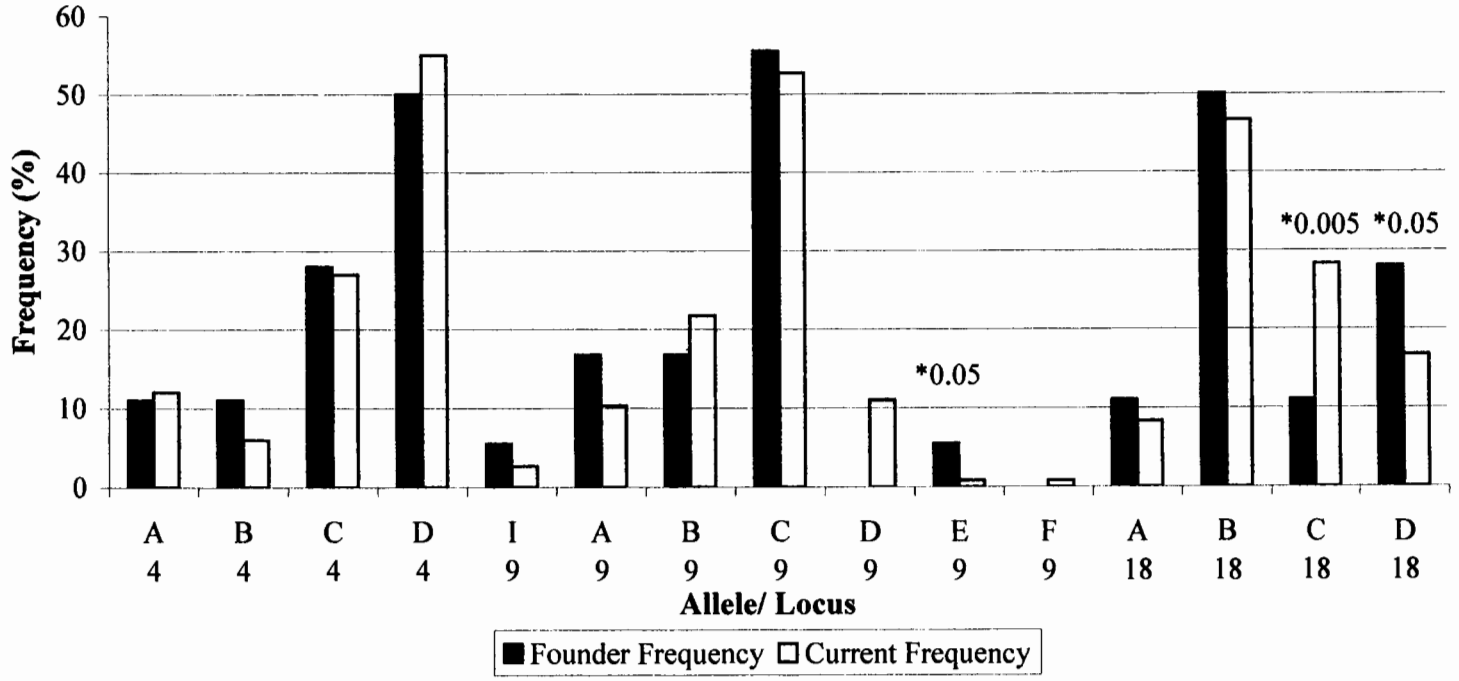

Fig. 3.4 Comparison of allele frequencies in the founding and current population. There has been a significant change in the allele frequency of 3 alleles at 2 loci between the founding population and the total 1998 captive population of *E. helvum.* Allele frequencies were calculated using CERVUS.

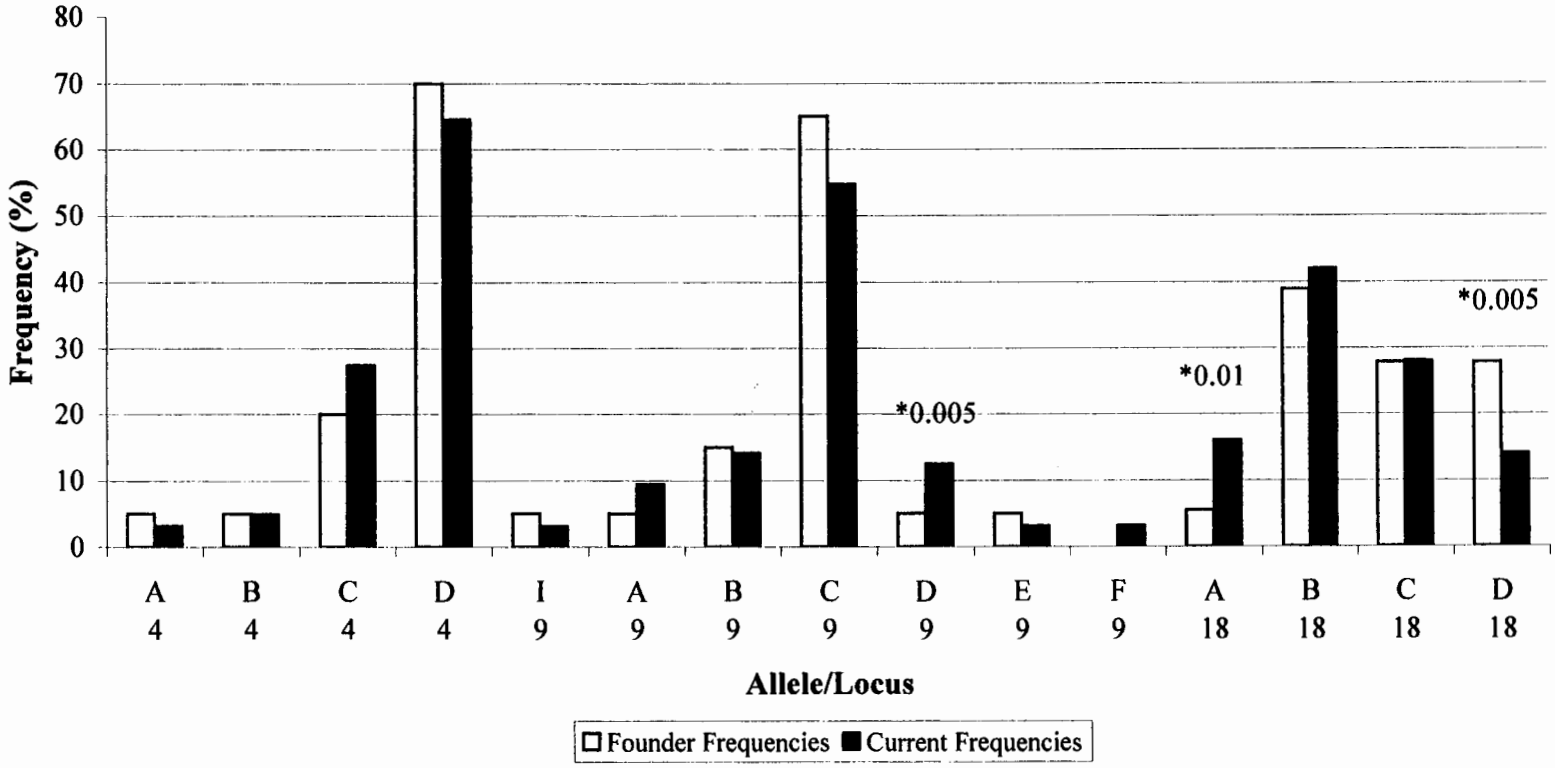

Fig. 3.5 Comparison of allele frequencies in the current and founding Portland colonies. There has been a significant change in allele frequency for three alleles at two loci between the Portland 1991 founding colony and the 1998 captive colony of *E. helvum*. Allele frequencies were calculated using CERVUS.

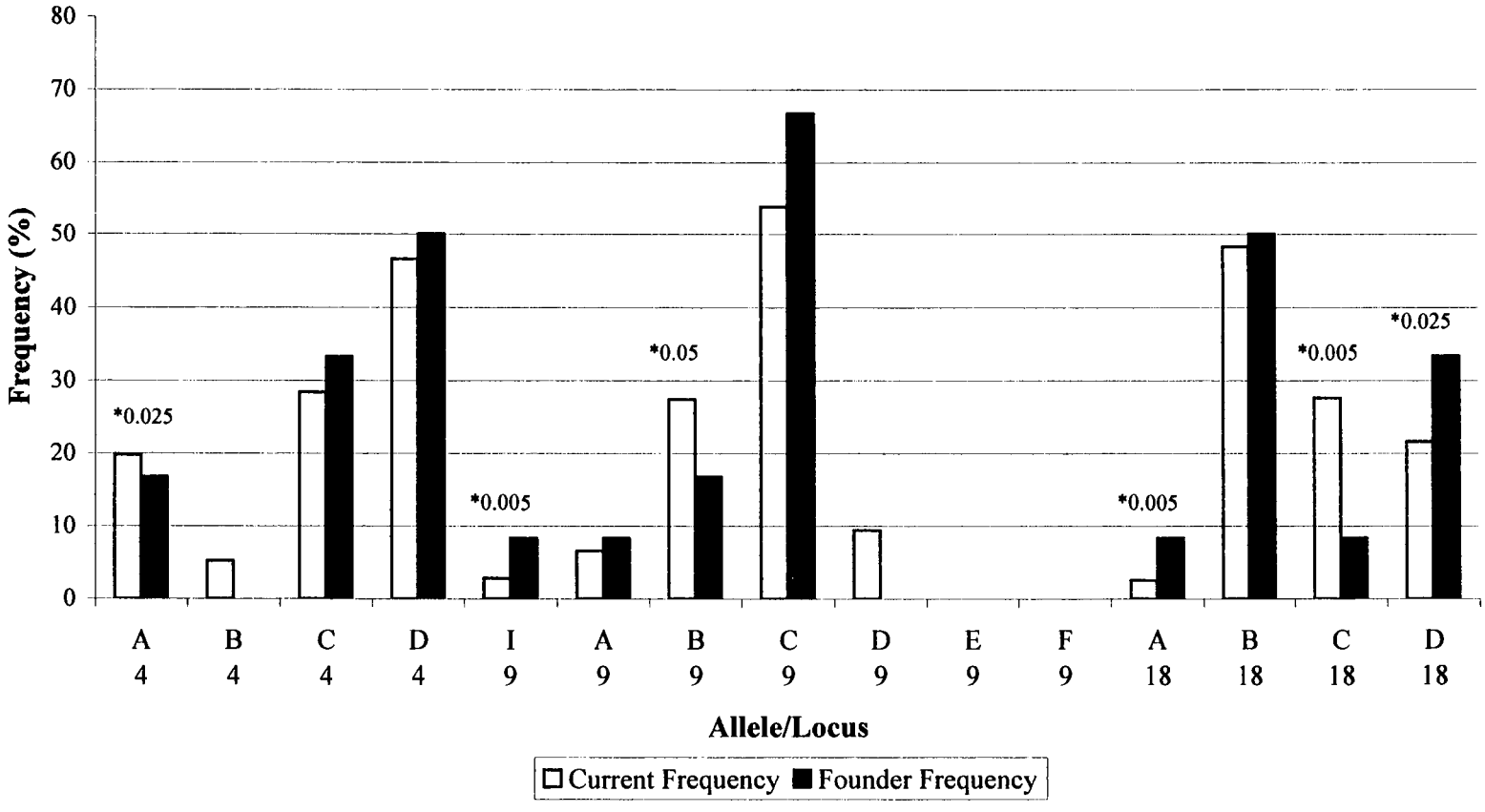

Fig. 3.6 Comparison of allele frequencies in the current and founding Milwaukee colonies. There has been a significant change in allele frequency at each of the three loci between the Milwaukee 1984 founding colony and the 1998 colony. Allele frequencies were calculated using CERVUS.

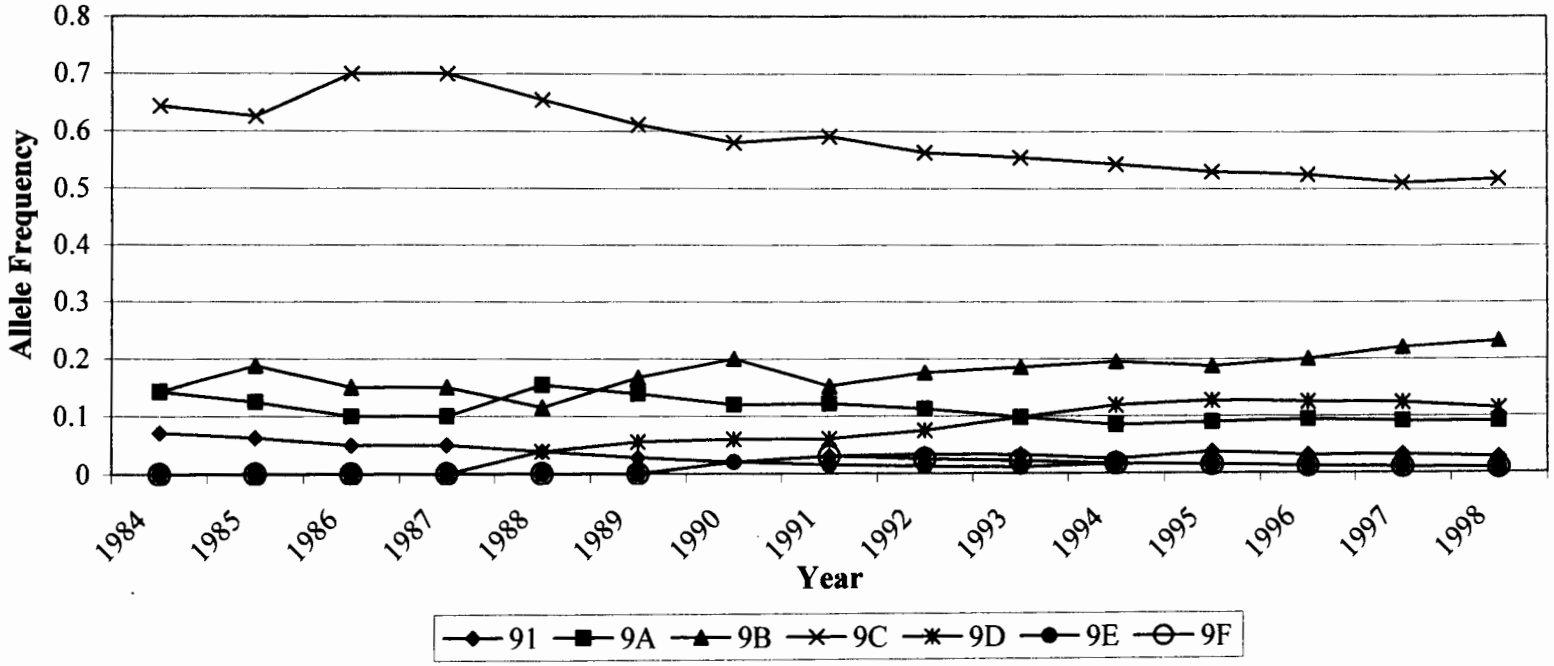

Fig. 3.7 Allele frequencies over time at locus P-9 for the captive population of *E. helvum*. This graph illustrates that there has been no loss of alleles at this locus since the founding of the population. Allele frequencies have been relatively stable over time. Alleles 9E and 9F have nearly identical allele frequencies. The first appearance of allele 9D in 1988 is most likely due to unsampled individuals present before 1988. Allele 9E was added to the population when a wild-caught male was imported to the Portland colony in 1990. Allele 9F, which appears for the first time in 1991, is a novel allele and is the only observed microsatellite mutation in this study. Allele frequencies were calculated using CERVUS.

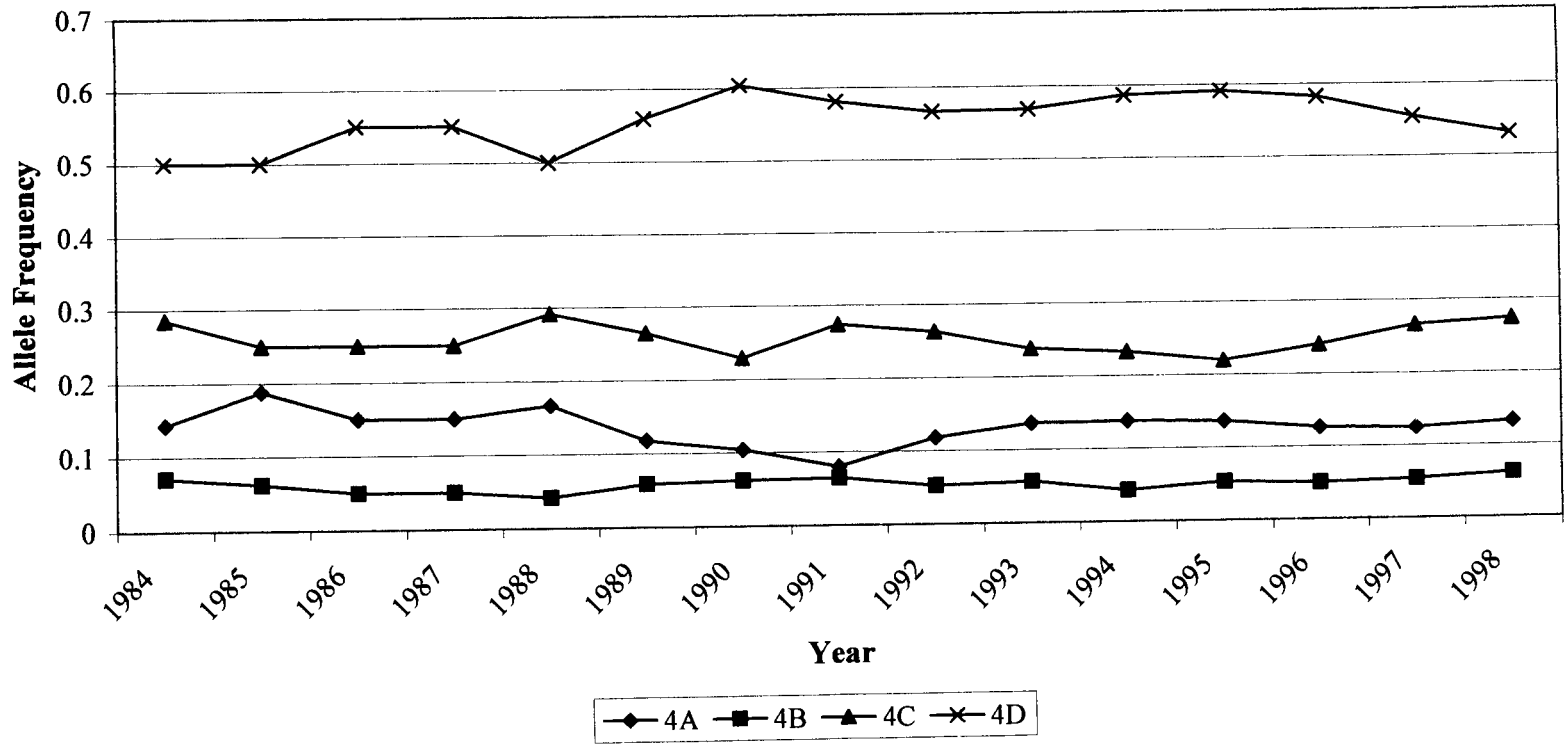

Fig. 3.8 Allele frequencies at locus P-4 over time in the captive population of *E. helvum*. This graph illustrates that there has been no loss of alleles at this locus since the founding of the population. Allele frequencies have been relatively stable over time. Allele frequencies were calculated using CERVUS.

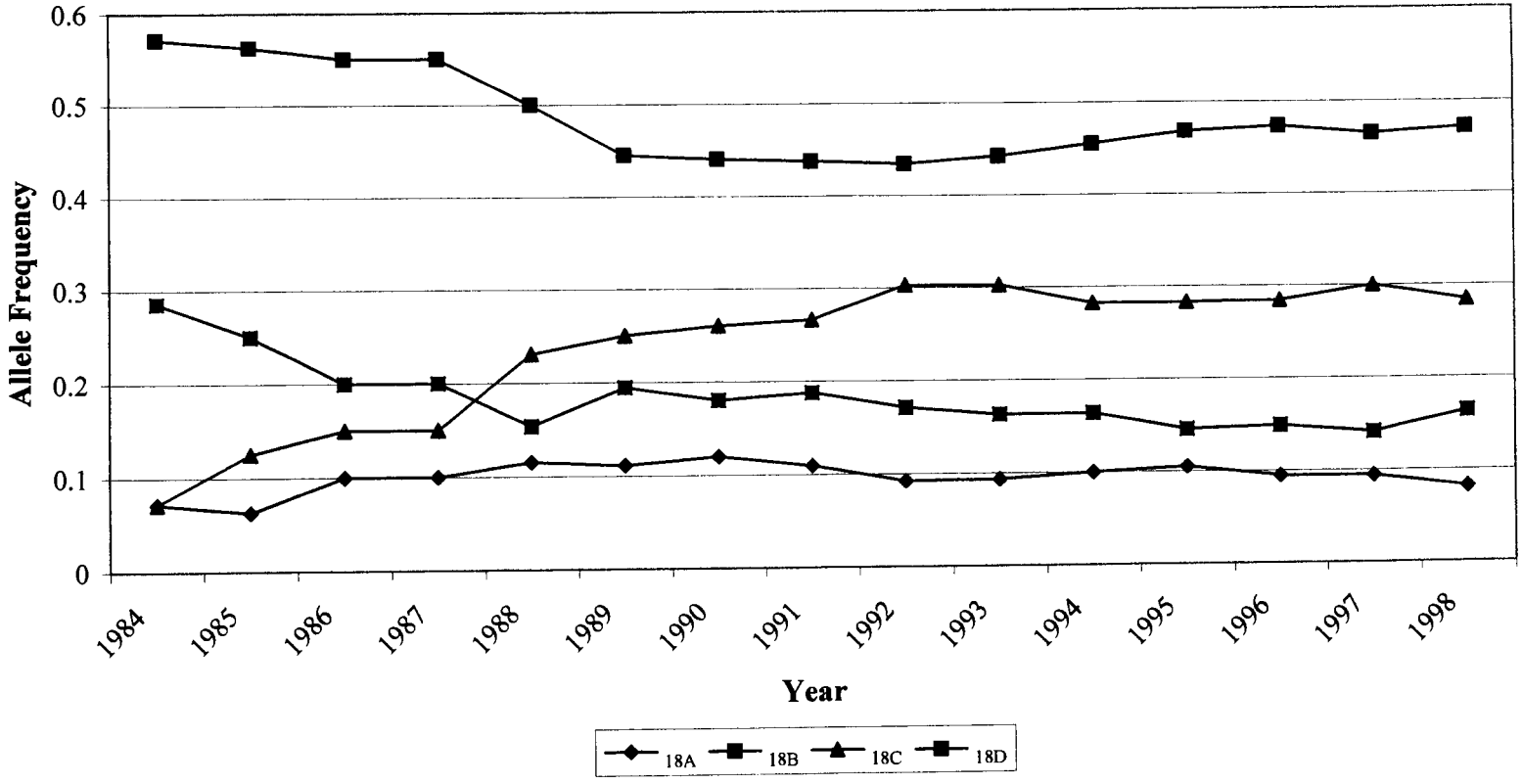

Fig. 3.9 Allele frequencies at locus P-4 over time in the captive population of *E. helvum*. This graph illustrates that there has been no loss of alleles at this locus since the founding of the population. Allele frequencies have been relatively stable over time, with most of the change occurring within the first 5 years after founding. Allele frequencies were calculated using CERVUS.

Chapter 4: Discussion

Genetic Variability in *Eidolon helvum*

In small populations, heterozygosity and allelic diversity tend to be lost through genetic drift (Wright, 1969; Wright, 1978). There is a correlation between heterozygosity and allelic diversity, but it is not absolute. Although maintenance of heterozygosity is thought to correlate with retention of quantitative genetic diversity, rare alleles may be lost at a higher rate than heterozygosity and therefore should be considered separately (Ballou and Foose, 1996). For example, it has been noted that loss of rare alleles may not necessarily have an effect on heterozygosity (Fuerst and Maruyama, 1986). Allendorf (1986) stated that rare alleles ($p<0.01$) are very likely to be lost during a bottleneck, such as in a founding event, with little effect on heterozygosity. With a founder size of 11 for the captive population of *E. helvum*, only alleles with frequencies greater than 0.3 would be likely to be retained with 95-99% certainty (Ballou and Foose, 1996). However, only two founders have been lost from the population, with one of these leaving 11 living descendants, and the majority of the diversity present in the founders has been retained, including many alleles with a frequency < 0.3.

Heterozygosity in the captive population of *E. helvum* was not statistically different from expected for any allele from any locus. In fact, because only 2 of the 7 sampled founders that were present before 1991 were heterozygous at microsatellite locus-P-18, there was a sharp increase in heterozygosity at this locus. Between 1984

and 1989 all loci converged toward a heterozygosity of approximately 0.6 and then stabilized. The change in heterozygosity each year during this early period was greater than the change in subsequent years. Heterozygosity is expected to be lost at a rate of 1/2N per year, where N is the number of individuals in the population (Wright, 1969). For example, between 1984 and 1986 the Milwaukee subpopulation had 3 males and 4 females, so heterozygosity in this colony was expected to be lost at a rate of 0.071 per year. In this same colony between 1987 and 1989 there were 2 males and 4 females, and heterozygosity was expected to be lost at a rate of 0.083 per year. However, the observed change in total heterozygosity during this time was actually an increase of 0.01. This increase in total heterozygosity was a result of an increase in heterozygosity at locus-P-18 of 0.27, and of decreases at loci P-9 and P-4 of 0.1 and 0.12, respectively. More importantly, heterozygosity at all loci stabilized within 5 years, and has remained stable. The captive population of *E. helvum* was able to avoid a loss in heterozygosity because of continued founder contribution to the population gene pool, overlap of generations allowing many years of founder/founder matings.

Maternity and Paternity Assignment

The combined exclusionary power of the three variable microsatellite loci used in this study was 0.55 for the first parent and 0.78 for the second parent (Table 3.8). This exclusionary power allowed for maternity to be directly assigned to 79.5% of the total offspring in this study, and for paternity to be assigned, at varying levels of confidence, to 69.6% of the total offspring in this study. Although CERVUS was

designed for use with wild populations, this study has shown that it is useful in studies of captive populations as well. In fact, with studies of captive populations, the assignment of most-likely sires can be critically evaluated using zoo records, further strengthening confidence in the assignment of paternity. Captive studies have the added advantage of knowing all possible candidate males, which allows complete confidence in the assignment of paternity when all but one candidate has been excluded. This is not the case for wild population studies, where not all possible candidate males are always known. In this study, CERVUS provided information about most-likely parentage, which was further screened using zoo records to provide the best estimate of parentage available from the current genetic data. The use of most-likely sires for demographic and genetic analysis is recommended until complete exclusionary data are available (Ballou and Foose, 1996).

Molecular methods that verify or assign parentage are especially important tools for identifying aspects of an animal's behavior and ecology that might otherwise go undetected. For example, twenty-one percent of the dams in this study were misidentified when using behavioral observations, suggesting that female *E. helvum* may be displaying complex parenting behaviors. A study of maternal behavior to investigate the root of these mismatches would be interesting. Information about paternity is also valuable for correlating mating behavior with mating success (Coltman et al.1999).

Additional variable loci would allow for the creation of a more accurate pedigree for the captive population of *E. helvum*. First, in recent years the number of

individuals descended from female founder #12 has increased sufficiently in the Milwaukee subpopulation to make maternity assignment not fully exclusive with the existing microsatellite loci. Additional loci would enhance exclusionary power in cases such as these where there are several closely related candidate dams. Secondly, additional loci would help determine whether male founder #32 has additional offspring. Currently there is only one confirmed and one suspected offspring for #32. However, some or all of #65's offspring may have actually been sired by #32. The current assignment of paternity using CERVUS indicates that the likelihood #65 sired these pups was greater than 90%. Additional variable microsatellite loci would confirm or refute this finding. Thirdly, although 27% of the candidate sires were not available for genetic testing, additional variable loci would increase the confidence in assignment of parentage to available sires. Furthermore, as these colonies continue to grow, it is recommended that additional microsatellite loci be used to improve the existing pedigree and studbook.

Subpopulation Structure

Skewed sex ratios, intrasexual variance in reproductive success, natal philopatry, and territoriality work together to maximize genetic subdivision (Storz, 1999). Many of these conditions are imposed upon captive animals. Their natural dispersal patterns are impeded, and if only one female line is present, then female philopatry is also imposed. The captive population of *E. helvum* has not shown a trend towards genetic subdivision between subpopulations. The microsatellite loci used in

this study show that there has been little genetic differentiation (F_{ST} = 0.003-0.03) between the subpopulations of *E. helvum*. F_{IS} and F_{IT} are not statistically different from zero, indicating that there is neither more nor less heterozygosity than expected (Tables 3.8 and 3.9). Promiscuous mating, existence of multiple female lines in many colonies, equivalence of litter size between females, exponential growth of the population, and occasional "migration" of animals between subpopulations appear to have contributed to the maintenance of heterozygosity in this captive population of bats. Continued exchange of individuals between institutions should be sufficient to maintaining genetic diversity.

When genetic drift occurs in small populations, the alleles fixed in each subpopulation may be different. It is therefore theorized that if separate subpopulations are maintained, a greater total genetic diversity will be preserved through the fixation of different alleles in each subpopulation (Foose et al., 1986). Foose et al. (1986) also suggested that the benefits of one large panmictic population can be attained with a migration rate of only one individual per generation. He proposed that a balance be achieved where subpopulations are large enough to avoid the effects of inbreeding depression and small enough to capitalize on the benefits of subpopulational differences. Foose et al. (1986) recommended subpopulations with greater than 25 breeding individuals, and an immigration rate of less than one individual per generation in order to achieve this balance.

Hardy-Weinberg Disequilibrium

Hardy-Weinberg disequilibrium was detected at locus-P-9 for the total population, and at locus-P-4 and locus-P-9 in the Milwaukee and Portland subpopulations respectively. In all cases, this disequilibrium appears to be due to an excess of heterozygotes. This excess in heterozygosity is not enough to be detected by the fixation index.

Founder Contributions and Pedigree Analysis

In this study, the requirements for being labeled a "founder" include having no known ancestors in the population, and either having descendants in the population or being capable of reproduction. Founders are, therefore, treated as unrelated individuals. This assumption would seem likely to be true in the case of these *E. helvum* because many of the founding individuals were captured in different regions of Africa in different years. There are, however, some cases that suggest the assumption of unrelatedness may not be true for all the founders. For example, female founders #13 and #5 share the same mtDNA haplotype. These two female founders also share all alleles at the microsatellite loci. They may, therefore, be closely related. Male founder # 8 shares the same haplotype as female founder # 12, and also shares one allele at each microsatellite locus. Since they were collected at the same time, #8 and #12 may also be closely related. For the remaining founders, the assumption of unrelated founders is supported by differences in mtDNA haplotypes and microsatellite alleles.

Complete descendant lists, including all living and dead descendants, were compiled using SPARKS and the microsatellite and mtDNA genetic data. Descendant lists were created for all female founders (Appendix D). The descendant lists also include individuals from the historical population that were not available for this study (gray shading). For all individuals available for this study, parentage assignments are based on genetic analysis. Parentage assignment for individuals that were not included in this study are based on zoo records.

The number of living descendants for each founder is illustrated in Fig. 4.1. Dam #13 has nearly twice as many living descendants as the other female founders, even though all other female founders, except #20, have been in the population as long or longer than dam #13. Dam #20 founded the Portland subpopulation six and a half years after dams #5, #6, and #12 founded the Milwaukee subpopulation, and 11 years after #2 founded the Seattle subpopulation. Male founders # 7 and #8 both have over 20 living descendants. Sire #32 has one confirmed and one additional suspected offspring. Male founder #10 was in the Milwaukee subpopulation for only two years before being removed to a private collection. Although he does not have any confirmed offspring, it is possible that he sired at least one offspring in those two years. Male founder #1 and female founder #2 were brought into the Seattle subpopulation in 1979. Many of their early offspring were either transferred out of the captive population or died, which explains the reduced number of living descendants from these founders, in spite of their length of time in captivity.

GENES was used to evaluate founder contributions to the current population using the pedigree constructed by SPARKS. Estimates of each founder's contribution, parity contribution, gene retention, target contribution, and evaluation of the status of each founder's contribution are found in Table 4.1. Founder contributions are calculated from the pedigree assuming the Mendelian premise that 50% of a parent's genes are passed to its offspring, 25% to grandoffspring, and proportionally less for each subsequent generation through the pedigree. The founder's genetic contribution to the total population is its contribution averaged across all individuals. Founder contributions for this population range from 0-0.19. Parity of contribution between founders would be achieved if each founder line had an equal contribution of 0.0909 of the total offspring.

Analysis of the pedigree and founder contributions by GENES shows that most founders should have near-complete retention of their alleles at all loci. Using the probable genetic contributions from a parent to an offspring, sire #32 has only 50% retention with one offspring, but may have up to 75% if the suspected second offspring can be confirmed. Because there are no confirmed offspring for male #10, his contribution, representation, and retention are listed as zero. As mentioned previously, assuming no offspring for this founder may be overly conservative.

Target founder contributions are in proportion to the retention of genes for each founder. As long as an individual is of reproductive age and remains in the population, there is the possibility for complete retention of genes. The target contribution for all founders is equal, which assumes that founder #32 will continue to

produce offspring and that founder #10 actually contributed offspring before his removal. If either of these conditions is not met, target contributions should be adjusted in proportion to the estimated retention of alleles for each founder line.

Retention of founder alleles, gene diversity, and founder genome equivalents were calculated using GENES through a gene drop analysis. For the gene drop analysis, each founder was assigned two unique alleles. Ten thousand simulations were run in which alleles were passed, using Mendelian inheritance patterns, from founders down through the *E. helvum* pedigree. After all simulations were completed, the fraction of founder alleles that were present in at least one copy in the current population was considered the mean allelic retention. Despite the assumption that male #10 has not contributed any offspring, there has still been a mean retention of 0.95 in the current captive population of *E. helvum*.

The founder genome equivalent is the number of founder genomes, assuming equal founder representation and no loss of alleles, required to obtain the same level of genetic diversity as the current population. Founders were excluded from analysis when determining the founder genome equivalent because they necessarily have 100% retention. For the captive population of *E. helvum*, the founder genome equivalent was 6.393, compared to the actual founder number of 11.

In summary, the current captive population of *E. helvum* has retained 95% of the original founder genetic diversity, and did so with the genetic equivalent of 6.4 founders.

Inbreeding coefficients and mean kinship values for each individual were also calculated using GENES and are given in Appendix E. Inbreeding reduces heterozygosity, and therefore, the inbreeding coefficient (F) is expressed as the fractional reduction in heterozygosity across all loci relative to the mean expected heterozygosity for a random mating population with no inbreeding. Inbreeding coefficients in this study ranged from 0 to 1, with 92% of the bats having an inbreeding coefficient of less than 0.25 (Table 4.2). However, caution must be used when evaluating the individuals in the 0 – 0.25 range, because 53% of the individuals in this range had one unknown parent, and because of this their genetic contribution to their offspring was omitted by GENES. A complete pedigree is needed to fully characterize the distribution of inbreeding coefficients for the captive population of *E. helvum*.

Mean kinship is the average relatedness of an individual to all other individuals in the population. These values are also biased by the omission of genetic contribution from unknown parents. Mean kinship values were calculated using GENES, and ranged from 0 –0.1182, with 23% of the individuals falling below 0.058 (Table 4.3). Offspring objectives are the total number of offspring suggested for an individual within a specified mean kinship range. The mean number of offspring from Table 4.3 corresponds to the mean number of offspring currently produced by the captive population of *E. helvum*. Therefore, the suggested offspring objectives would not alter the current population growth.

Genetic diversity is directly related to mean kinship and to founder genome equivalent, as shown by the following equations:

$$\text{Genetic diversity} = 1 - mk$$

Or

$$\text{Genetic diversity} = 1 - 1/2f_g$$

where mk is mean kinship and f_g is founder genome equivalent (Ballou and Lacy, 1995). Population management strategies that work to equalize founder contribution and allelic parity in the captive population of *E. helvum* would increase the founder genome equivalent of 6.393 towards the actual number of founders. Founder alleles can be lost either by genetic drift or unequal founder contribution. Because there is no evidence that genetic drift has played a significant role in the captive population of *E. helvum*, the majority of the disparity between the number of founders and the founder genome equivalent can simply be attributed to skewed founder contributions. Because nine of the eleven founders remain in the population, appropriate management strategies could still balance founder contributions. A detailed discussion of management options for restoring equivalence of founder contribution is offered later.

Mating System

Genetic and behavioral observations suggest that captive *E. helvum* have a promiscuous mating system. There is no evidence for mate fidelity or polygyny in the observed pedigree.

Demographic Analysis

As a result of the genetic analysis that determined parentage for the offspring in this study, a pedigree was constructed and a demographic analysis of the captive bat population was performed.

The fact that original founders are still alive and reproducing complicates the demographic analysis. Life span, mortality, and net reproductive rate can only be estimated at this time. For captive subpopulations of *E. helvum*, where nine of the founders are still reproducing, this may mean that more than 30 years of demographic data will be required to fully characterize lifetime reproductive contributions of individual bats to the population. Demographic studies that span a time less than the life-span of the organism may not accurately describe reproductive contributions of individuals (Schwartz et al., 1998). For *E. helvum*, this means that ongoing analysis will be important to avoid potential population instability following the loss of important contributing founders.

Reproduction and Mortality

Females tend to have one offspring per year beginning at age 2, while males sire the majority of their offspring between the ages of 10 and 17 (Fig. 4.2). Although females appear to reproduce consistently throughout their lives, males reproduce over a slightly shorter range of years. However, male reproductive behavior as males age remains to be fully described. For males, some mating occurred as early as four years of age, but most successful matings occurred closer to age ten. Females, on the other hand, showed no age-specific peak in reproductivity. This indicates that management

decisions concerning reproductive males must take the age of the male into consideration. As more males in this population exceed age 15 it will be interesting to see if an upper age limit of reproductive success exists for male *E. helvum*. There has been no evidence to-date of senescence in males or females and the upper limit of age in captive *E. helvum* is unknown. The majority of deaths that have occurred were before the age of two, with 16% of these deaths occurring within the first 30 days after birth (Fig. 4.3).

Generation time represents the average age at which an animal produces an offspring. Based on genetic assignments and studbook records, SPARKS was used to calculate generation time (T). The captive *E. helvum* in this study have generation times of 10.17 years for males, and 8.58 years for females. Older founders who are still breeding bias both male and female generation times.

Population Growth

The product of survivorship X average number of offspring was calculated using SPARKS for each age class. The sum of these products is the net reproductive rate (R_o). R_o is the replacement rate for an individual, with $R_o < 1$ indicating a declining population, $R_o = 1$ indicating a stable population, and $R_o > 1$ indicating a growing population. To-date, males and females have equal longevity in captivity, and demonstrate similar net reproductive rates. With a net reproductive rate of approximately four, the captive population of *E. helvum* appears to be growing rapidly (Table 4.4). It should be stressed that values for longevity and net reproductive rate are biased because neither male nor female founders have stopped reproducing.

The change in population size can be expressed as the percent of population change per year (λ). If $\lambda < 1$ indicating a declining population, $\lambda = 1$ indicating a stable population, and $\lambda > 1$ indicating a growing population. For the captive population of *E. helvum*, $\lambda \cong 1.16$. This suggests that a high increase in population size may have contributed to the high retention of genetic diversity, heterozygosity, and founder genomes, since genetic diversity tends to be lost when growth rates are slow (Ballou and Foose, 1996).

The captive population of *E. helvum* has shown exponential growth over the past 20 years (Fig. 4.4). Since 1992, the male population has increased at a faster rate than the female population. This trend towards unequal numbers of male and female offspring, with a bias toward males, is of concern and should be further studied. Reduction of females, especially in under-represented founder lines, would strongly impact the risk of a decrease in genetic variability in the future. Whether the trend towards male offspring is by chance alone, or is the result of unknown environmental or biochemical factors would be interesting to investigate. Annual analysis is recommended to monitor this trend.

Effective Population Size

The effective size of the population (N_e) is the number of successfully breeding individuals. This number is important because, unlike the census number, it determines a population's genetic characteristics. It is, therefore, the population meaningful number when evaluating the effects of drift, inbreeding, and in calculating

the sustainability of the population. In this study, N_e was calculated using the equation:

$$N_e = (4\ N_m \times N_f)/ (N_m + N_f)$$

where N_m and N_f represent the number of successfully breeding males and females respectively. The current N_e and the ratio of N_e to the census number for each subpopulation is given in Table 4.5. In 1997, Milwaukee transferred six of its ten reproductive males to Lincoln Park Zoo's single-sex non-breeding colony. This caused a drop in effective subpopulation size for Milwaukee from 25.7 to 13.09. Effective population sizes usually represent approximately 30-50% of the census population (Ballou, 1987). Effective subpopulation sizes for captive *E. helvum* range from 22-69% of the census subpopulations' size, which is well within the range described by Ballou (1987). A low N_e/N ratio is expected for an exponentially growing population with many animals below reproductive age.

Carrying Capacity

For animals in captivity, the carrying capacity is the target population size. The carrying capacity of a captive population is affected by several factors, including the management goals for the species. For example, there is currently an interest in expanding the captive population of *E. helvum* as more zoological institutions design bat exhibits. These fruit bat exhibits are very popular with the public, and it is possible to house large fruit bat colonies in comparatively less space than is needed for other colonial, flocking, or herding animals. For these reasons, there is currently no indication for prohibiting the breeding of any of the individual bats. However, given

the longevity of these animals, careful planning is required to avoid overpopulation in the captive colonies. Until the zoos maintaining or planning to house *E. helvum* establish their projected population sizes, a carrying capacity cannot be determined. However, consideration of factors that will influence the carrying capacity of *E. helvum* is very useful. By carefully considering these influencing factors, a plan for reaching a reasonable carrying capacity and stabilizing the population can be made which maximizes and equalizes maintenance of founding genetic variability. It is certainly possible that despite a founding population of only eleven individuals a genetically diverse, self-sustaining, captive population of *E. helvum* can be maintained.

Rapid population growth rate decreases the number of individuals required to maintain genetic variability. This is because more heterozygosity has been retained in the early years of breeding (Ballou, 1987). The captive population of *E. helvum* has certainly exhibited rapid population growth. The presence of overlapping generations also contributes considerably to the maintenance of variability seen in *E. helvum*.

It has been suggested that populations founded with fewer individuals require a larger carrying capacity than populations with greater founder numbers in order to retain an equal amount of the original heterozygosity (Ballou, 1987). This is because larger numbers of founders should more accurately reflect the heterozygosity from the wild or source population. However, it is actually the effective number of founders, often lower than the census number of founders, that must be considered (Foose et al., 1986). For *E. helvum*, there is reason to believe that all founders have contributed to

the current population, thus lowering the minimum carrying capacity required for a sustainable captive population.

Ballou (1987) showed that for species with non-overlapping generations, generation time is positively correlated with carrying capacity (Ballou, 1987). Under the condition of non-overlapping generations, a species with a short generation time will lose genetic variability more quickly than a species with a long generation time. This has not been the case for *E. helvum* given that there was extreme overlap of generations. The variability from the founders is still being introduced into the population. Therefore, with *E. helvum*, the more offspring produced, the greater the chance there will be maximum retention of the founder genes after the loss of the founders.

In general, the lower the fecundity or reproductive potential of a species, the higher the founder number needs to be, because of loss of diversity during expansion (Foose et al., 1986). Relatively high fecundity suggests that *E. helvum* fruit bats may have withstood the adverse effects of having had fewer founders better than other taxa with lower fecundity.

The introduction of additional founders is considered beneficial to counteract effects of genetic drift, and to capture additional alleles from the wild. The number of additional founders from the wild that are necessary to achieve these goals depends upon the management strategy (Ballou, 1987). If the intent is to maintain genetic diversity and preserve as much of the wild variation as possible, thereby keeping the captive population as a future resource for wild populations, intermittent immigration

of wild individuals is desirable. If, however, the population is to be used solely as a display species for exhibit purposes, then adaptation to captivity and loss of rare alleles is of minor consequence. In reality, for *E. helvum*, taking to heart the state of the "demographic winter" that humans are imposing on the environment (Soulé, 1986), it might be sensible to preserve as much genetic variability as is feasible under current financial and logistical restraints. Indeed, most captive propagation programs are faced with this need to balance the desired maintenance of self-sustaining populations with financial and logistical constraints (Foose et al., 1986).

Microsatellite and Mitochondrial DNA Mutation Rate

Mutations were observed for both the microsatellite loci and the mtDNA. With a proposed mutation rate of 10^{-2} to $5x10^{-6}$ per gamete per locus for microsatellites (Edwards et al., 1992; Hearne et al., 1992), and a rate of $10x10^{-9}$ substitution per nucleotide site per year for mtDNA (Hartl and Clark, 1997), some mutation would be expected in the twenty years encompassed by this study. Some interesting examples were:

1. Although dam #13 had haplotype II, all of her offspring had haplotype III. The pups were assigned to dam #13 because all other dams present in the Milwaukee subpopulation shared unique and matching haplotypes and genotypes with their own offspring. An additional discrepancy between dam #13 and her assigned pups occurred at microsatellite locus P-18. Dam #13 had the apparent genotype DD, but all of her pups were either heterozygous with a B allele, or homozygous for B alleles.

This indicates either the possibility of a null allele at this locus for dam #13, or that she is mosaic for this locus. Because of these two discrepancies between dam #13 and her assigned pups, it would be useful in the future to isolate her before the birth of her next offspring and obtain a tissue sample from her offspring before introducing the pup to the colony. This would confirm maternity. In addition, necropsy samples from various tissues, including tissues from reproductive organs, should be obtained after her death to test the hypothesis that #13 is mosaic for different cell lines. At this time, however, assignment of these pups to #13 represents the best fit for the available genetic data.

2. There have been several mtDNA mutations since the founding of the captive population. In the Seattle collection in 1990, dam #2 had an offspring (#50) with haplotype I, although all previous and subsequent offspring assigned to dam #2 had haplotype IX. At the time of #50's birth, dam #2 was the only female of breeding age. This female died in 1996 and no genetic data are available to confirm a match using microsatellite loci. The two haplotypes present in the descendants of #2 indicate that either a mutation occurred in #50, or that dam #2 was also a mosaic for two different cell lines.

3. In 1998, two pups were born in the Milwaukee subpopulation (#216, #268) with the unique mitochondrial haplotype X. There were several pups born in Milwaukee in 1998 with unknown dams, so it was not possible to determine the origin of this mutation. Because there have been no records indicating the birth of twins, it is curious that there would be two pups born in the same year with the same mutation.

This suggests that the zoo records for the date of birth for these individuals may not be accurate or that the birth of twins was not observed. It was not possible to assign a specific dam to these pups with the current three microsatellite loci. The addition of more variable microsatellite loci would be of help.

4. In 1989, a male (#44) was born in the Milwaukee subpopulation with a mtDNA haplotype V. This haplotype was not present in the Milwaukee subpopulation, and in fact did not arrive into the total population until 1991 when a new wild-caught female (#20) founded the Oregon Zoo subpopulation. It is unclear at this time whether this represents a mutation, or if there is an error in the zoo records.

5. There is one example of a captive born offspring having a unique microsatellite allele. In 1991, a pup (#68) born to dam #20 had a genotype of FF at locus P-9. No founder, including #20, had the F allele. Several amplifications and gels were run for #68 to verify the presence of this unique allele. Because #68 is apparently homozygous for this allele, there was either a mutation at both the maternal and the paternal loci, or #68 has one null allele and one unique allele.

Microsatellite and mtDNA RFLP analysis have been shown to be useful in the captive population of *E. helvum* for determining parentage and relatedness. The examples of mutations within this captive population suggest that caution is warranted when using these techniques in wild populations. As is possible in the captive population of *E. helvum*, somatic cells may not always represent the genotype of germ cells. Nor is a difference in somatic and germ cells necessarily a one-time event. A mutation may be consistently passed from mother to offspring. The frequency of

mutation observed in this study supports the assertion by Dowling et al. (1996) that identical migration patterns of DNA should only be considered homologous for closely-related individuals. It is important to recognize that molecular tools used today detect genetic variability that was produced by a variety of mutational mechanisms. Also, the degree of variability found using a particular technique is related to the rate of mutation expected for the region of DNA being investigated.

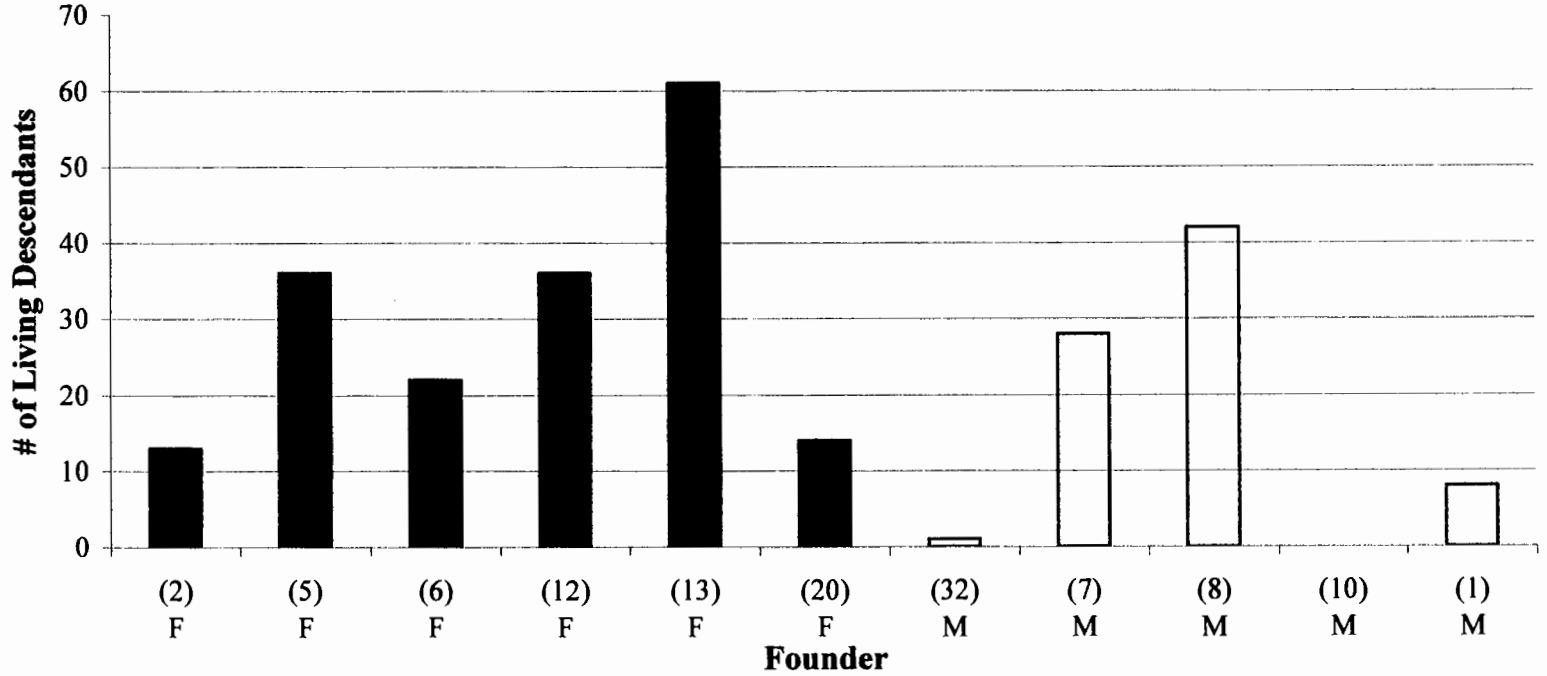

Fig. 4.1 Founder contributions in number of living descendants for each wild-caught founder of the captive population of *E. helvum*. Founders are designated by their studbook nnumber. The sex of the founder is indicated as female (F, shaded bars) or male (M, open bars). Founders #1 and #2 were imported in 1978, founders #5, #6, #7, #8, #10, #12, and #13 were imported in 1984, and founders #20 and #32 were imported in 1990. Nine of the eleven founders remain in the breeding population. Founder #2 died in 1996, and founder #10 was removed from the population in 1986. Founder contributions were calculated using SPARKS.

Table 4-1 Summary of Founder Contributions

Founder	Current Founder Contribution to the total population	Parity Founder Contribution	%Genes Surviving (retention)	Target Founder Contribution	Status of Contribution
1	0.0457	0.0909	0.993	0.09094	Under
2	0.0639	0.0909	0.996	0.09060	Under
5	0.1301	0.0909	0.998	0.09094	Adequate
6	0.0852	0.0909	0.996	0.09094	Under
7	0.1001	0.0909	0.999	0.09094	Adequate
8	0.1895	0.0909	1	0.09094	Over
10	0.0000	0.0909	0	0.09094	Under
12	0.1210	0.0909	0.999	0.09094	Adequate
13	0.1900	0.0909	0.998	0.09094	Over
20	0.0685	0.0909	0.998	0.09094	Under
32	0.0061	0.0909	0.5	0.09094	Under

Founders are indicated by their studbook number. The current founder contribution to the total population for each founder is its average contribution across all individuals calculated from the pedigree. This calculation assumes the Mendelian premise that 50% of a parent's genes are passed to its offspring, 25% to its grandoffspring, and proportionally less for each subsequent generation through the pedigree. Parity contribution for each founder is the proportion of genetic contribution to the population required from each founder for all founders to equally contribute. Retention is fraction of the founders genes retained in at least one copy in the population as estimated by gene drop analysis. Target founder contribution is proportional to the founder's retention. Any founder remaining in the population and capable of breeding will have a target contribution equal to those who have already contributed. The status of a founder line's contribution is determined by comparing the target to the observed contribution. These calculations were performed using GENES.

Table 4-2 Inbreeding Coefficients

Range	Percent of Population
0.0-0.25	96*
0.26-0.5	3.2
0.51-0.75	0
0.76-1	0.6
Mean Inbreeding Coefficient	0.013

* 53% of individuals in this category have 1 unknown parent. These calculations were performed using SPARKS.

Table 4-3 Mean Kinship Values and Offspring Objectives

Mean Kinship Range	% of Population	Offspring Objectives
<0.019	1	6
0.020-0.034	5.3	5
0.035-0.050	7	4
0.051-0.058	9.7	3
>0.058	77	2
		Mean = 4 offspring

Offspring objectives are given for individuals in each mean kinship range. The average offspring objective corresponds to the observed average number of offspring being produced by an individual in the captive population of *E. helvum*. These calculations were performed using SPARKS.

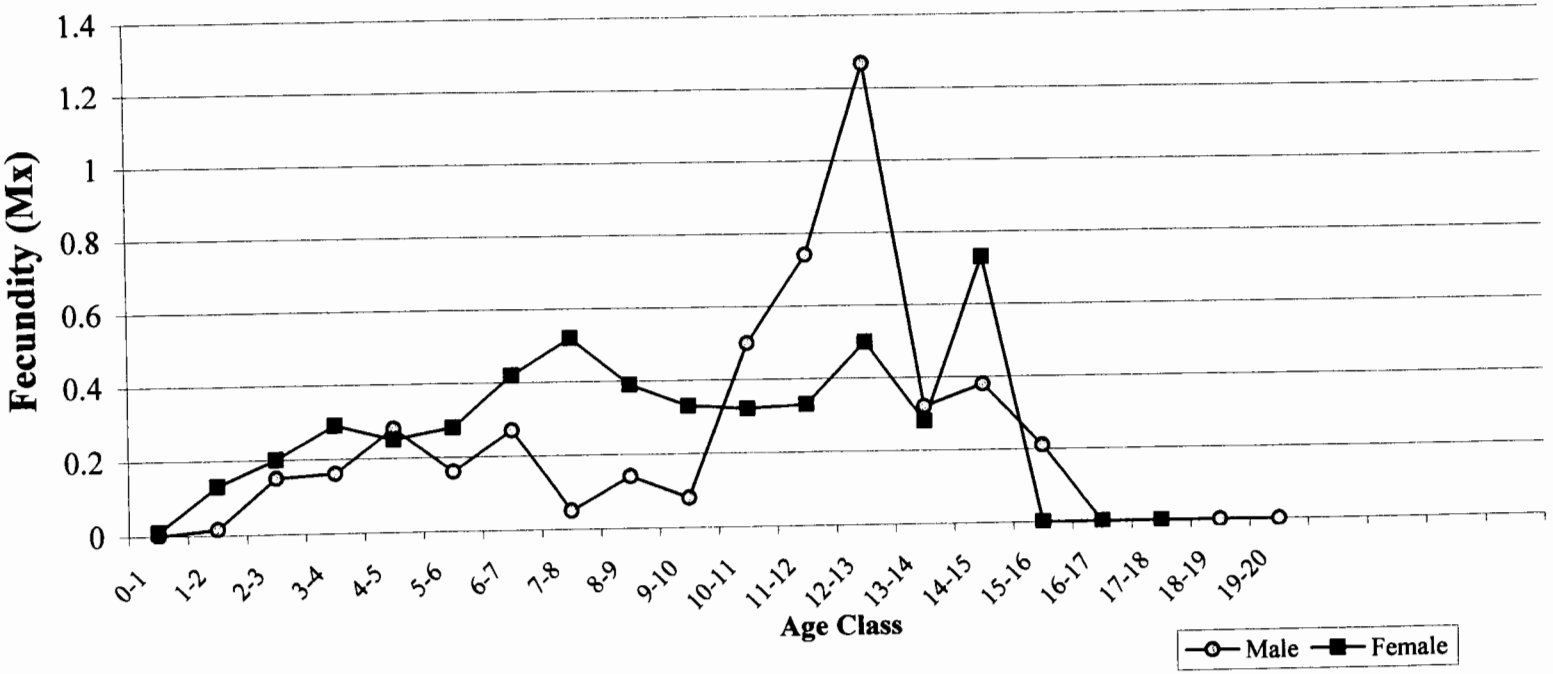

Fig. 4.2 Fecundity (Mx) for the captive population of *E. helvum*. Mx is calculated as the ratio of the number of births credited to an age class to the number of individuals that have passed through the same age class. More analysis is needed for individuals older than 16 years of age. There were only one to two individuals of each sex in this age class. Therefore, the drop in fecundity above age 16 should not be considered senescence. Females tend to produce their first young at age two and continue to produce one young per year there after. Males have an age specific peak in reproduction between 10 and 15 years of age. Fecundity data was calculated using SPARKS.

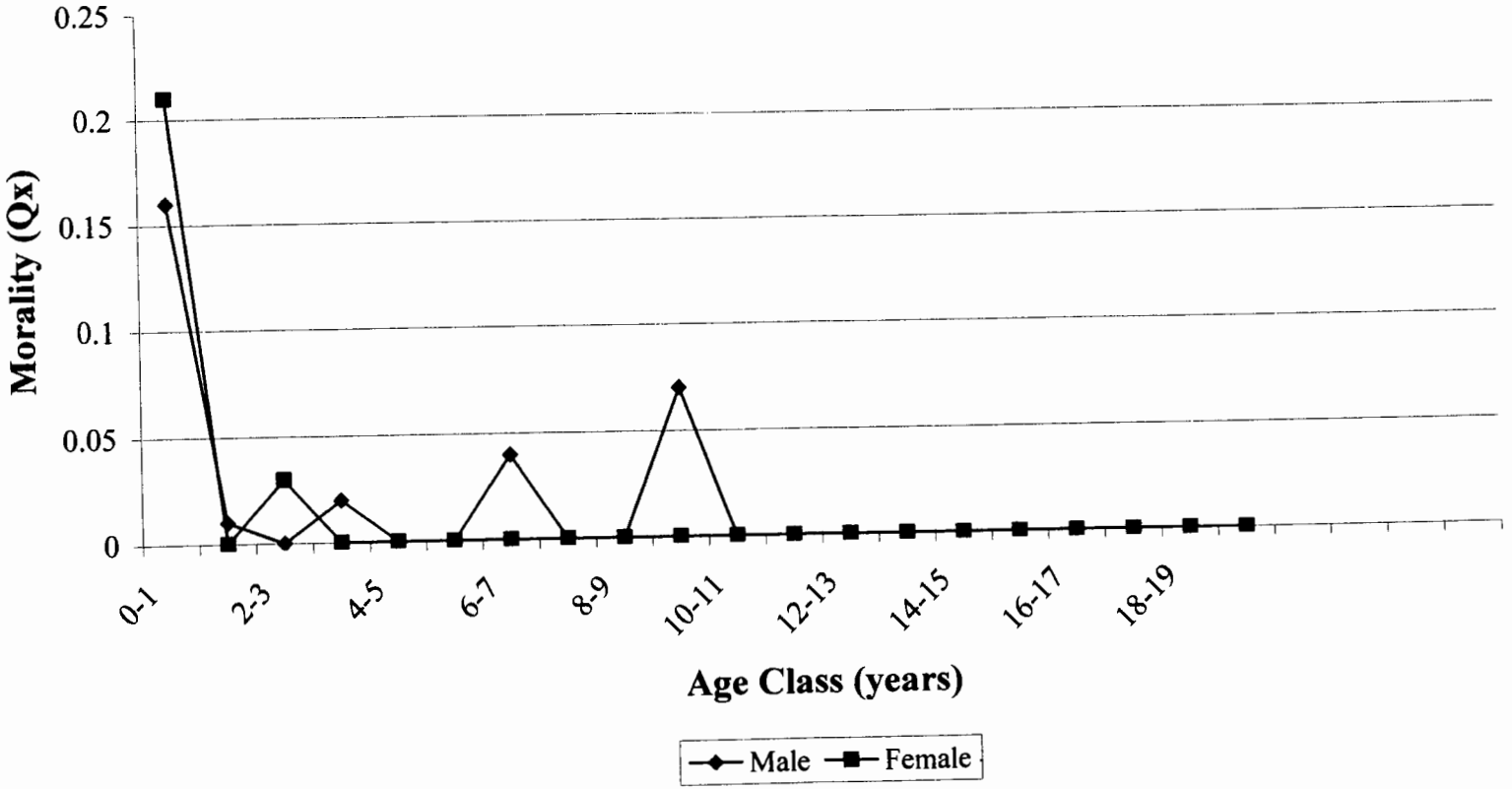

Fig. 4.3 Mortality (Qx) for each one year age class in the captive population of *E. helvum*. Qx is calcualted as the ratio of the number of deaths in an age class to the risk of death for an age class. This graph illustrates that *E. helvum* are a long-lived species, with the majority of deaths being attributed to the 0-1 and 1-2 age classes. Mortality was calculated using SPARKS.

Table 4-4 Fecundity Statistics

Statistic	Males	Females
T (Generation Time)	10.126 Years	8.576 Years
Ro (Net Reproductive Rate)	4.063	3.820
Lambda (λ) (Population Growth Rate)	1.15	1.17

Generation time is the average age at which an animal produces its offspring. Ro is the replacement rate for an individual (Ro>1 = a growing population, Ro<1 = a declining population, Ro = 1 = a stable population). $\lambda$is the percent change in population size per year (λ>1 = a growing population, λ<1 = a declining population, λ = 1 = a stable population). These calculations were performed using SPARKS.

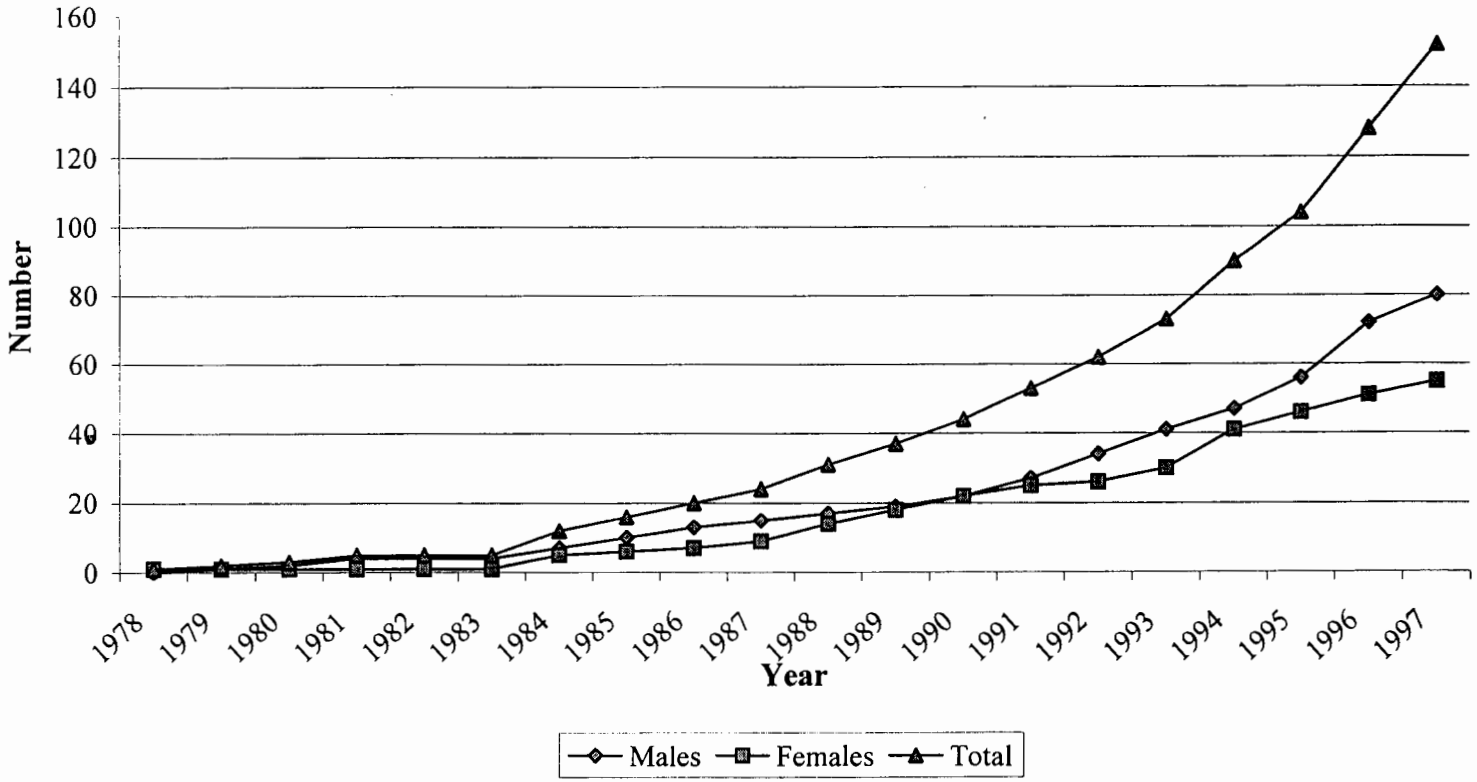

Fig. 4.4 Census population each year for the captive population of *E. helvum*. The total census population as well as male and female portions of the population are given. Since 1991 more male offspring than female offspring have been produced. The difference between the total census and the sum of the male and female census numbers can be attributed to individuals of unknown sex included in the total census count. The captive population of *E. helvum* has displayed exponential growth. Census data was calculated using SPARKS.

Table 4-5 Effective Number of Individuals at Each Institution in 1998

	Milwaukee	Portland	Seattle	Franklin Park
N_e, 1998	13.09 *(25.7)	20.57	4.8	7.5
N_e/N	.218 *.389	.502	.686	.577

*N_e before the transfer of 6 males to Chicago
N_e defines the number of successfully breeding individuals. N_e/N describes the proportion of each subpopulation that are included in the effective number. N_e/N is typically 30-50% of the census number. In the case of the captive colonies of *E. helvum*, the majority of individuals in the census that are not included in N_e are juveniles.

Chapter 5 Specific Breeding Recommendations and Conclusions

Management and Breeding Recommendations

E. helvum bats breed well in captivity and are not currently threatened in the wild. However, caution is warranted when evaluating the status of *E. helvum* bats in the wild since they are used as a food source, are often considered pests, and they rely on what is now declining habitat. As there is currently no basis for advising reintroduction of captive individuals to the wild, adaptation to captivity is considered an acceptable objective for the captive population of *E. helvum* (Ballou, 1987). The present captive management goal for this species is to maintain self-sustaining breeding colonies with some non-reproducing groups used for display and educational purposes only. The information obtained through this study was used to describe the mating system of *E. helvum* and the genetic and demographic constitution of the current population. This information makes it possible to form specific recommendations for the future management of this species in captivity.

Population management decisions strive to maintain genetic diversity, and decrease inbreeding and mean kinship. This works to avoid inbreeding depression, and to maximize the response to selection. Consequently, the management decision recommendations from this study emphasize ways to maintain genetic diversity and allelic parity among founding lines.

Management recommendations that increase the founder genome equivalent or decrease mean kinship values will increase genetic diversity. Increasing the effective

population size will minimize the effect of drift and help to preserve both heterozygosity and allelic diversity (Allendorf, 1986). It should be noted that morphological or reproductive aberrations should be selected against, even at the loss of genetic diversity.

Pfeifer (1996) suggested that decisions about dispersal of individuals between institutions should be based on the mating system and social structure. But, both genetic and demographic information are important when choosing individuals to be transferred from one institution to another. When transferring bats between colonies, the inbreeding coefficient, mean kinship, and reproductive age of the individual should be considered, as well as an evaluation of the relatedness and reproductive status of the remaining bats in the donor breeding colony.

Although the breeding subpopulations of *E. helvum* currently retain a high percentage of their allelic diversity, and have relatively low inbreeding coefficients and mean kinship values, some changes could be made to maintain this trend and to encourage greater allelic parity among founding lines. Allelic parity results when alleles from all founding lines are represented equally. However, striving for equal founder-line representation in the current population of *E. helvum* is not reasonable unless there has been an equal numbers of alleles retained from each founder line. Target contributions should be proportional to the percentage of the founder's genome that has been contributed to the current population relative to other founders' contributions. For example, if male founder #32 were removed from the population after contributing only one offspring, his target contribution would be half that of

founders with two or more offspring. This is because he would have a retention of 50%, and would be essentially one-half a founder (Ballou and Foose, 1996). Giving equal contribution to founders that have not passed on equal proportions of their genome over-represents the portion they have contributed (Ballou and Foose, 1996). Target founder contributions were calculated as the fraction of each founder's genome that was retained. Target founder contributions in this study were equal for all founding individuals, which assumes all founders of the captive population of *E. helvum* have contributed an equal proportion of their genome. Population management goals to equalize target contributions are reasonable for this population only if male founder #10 can be shown to have actually contributed two offspring, as is suspected, and male #32 continues to contribute offspring to the population. If these conditions are not met, male founder line #32 should have a target contribution half that of the remaining founders. Male #10 would not have a target contribution because he would not meet the definition of a founder if he did not contribute any offspring to the population. All remaining founders either have had multiple offspring or remain in the population and have the opportunity to reproduce.

Specific management recommendations based on the results of this study are detailed in the following section. A summary of suggested animal transfers is provided in Table 5.1.

1. In order to maximize allelic parity among all founders, all descendants from the deceased female founder #2 should be maintained in breeding colonies. Only 11 of her 22 descendants are living, and of those only 2 are females. Three of her

descendants, all males, are currently being housed in non-breeding colonies. Of these three, the transfer of #116 to a breeding colony would have the greatest immediate impact on retaining founder #2's alleles since he is just approaching reproductive age. The other two males are over 14 years old and would be expected to contribute fewer future offspring. Both females are currently housed in the Oregon Zoo colony, and both are breeding. Jacksonville also houses a male descendant of #2, and although there are 2 females in the colony, there are 4 additional males of similar age, lowering his chances of successful mating.

2. Seattle's subpopulation no longer has males or females from the original female founder #2. Currently, its only females are descended from Milwaukee's female founder #6. These animals are breeding well, but, as their offspring reach reproductive age, they will be forced to inbreed. Only one male, less than 3 years old, represents the founder line #6 in the Portland subpopulation. Transferring offspring from the Seattle subpopulation to Portland in exchange for individuals descended from female founder #20 would solve both the potential inbreeding problem in the Seattle subpopulation and increase the distribution of founder line #20. Although female founder #6 is still present in the Milwaukee colony and continues to reproduce, she has no other female descendants in Milwaukee. In fact, the only descendants from founder line #6 remaining in Milwaukee are males under 3 years of age. The one male descendant from the Milwaukee subpopulation that was successful in mating was moved to a non-breeding colony at Chicago's Lincoln Park Zoo. Any descendants

from this line should be maintained in breeding colonies, with preference given to re-establishing the line in Milwaukee.

3. As mentioned previously, descendants from female founder #20 exist in a breeding environment only in Portland. Long-term plans to expand this line to other breeding colonies would help to maintain allelic parity among the founders.

4. Male and female descendants from female founder #13 are present in both Milwaukee and Portland. Three females from this line were used to colonize the Portland subpopulation. This line is the most well represented and has been successful in both subpopulations. Female founder line #5 is also a well represented line in both of the larger breeding colonies, Portland and Milwaukee. Care must be taken to assure that female founder lines #2, #20, and #6 are not lost as a consequence of the success of these other lines.

5. Male founder line #1 faces similar problems as female founder line #2. Seven of his 11 descendants are either dead or in non-breeding colonies. Of the remaining four, two are in Seattle, one is in Jacksonville, and one is in Boston's Franklin Park Zoo. Because male founder #1 is still producing offspring, consideration should be given to transferring one or more of his offspring to breeding colonies such as Portland or Milwaukee.

6. Male founder line #8 is well represented in both Milwaukee and Portland. Five males from this line are currently housed in Chicago's non-breeding subpopulation. However, this is relatively inconsequential since other females and males of this line remain in breeding colonies.

7. Minimizing inbreeding coefficients and mean kinship values is also an important population management goal. Pertinent to this consideration, Milwaukee transferred six of ten males of reproductive age to the single-sex group at Chicago's Lincoln Park Zoo in 1997. Many of the males now housed in Chicago have low inbreeding coefficients. For example, males #25, #53, #73, #74, #75, #104, and # 130 have inbreeding coefficients of 0.0 with greater than 75% of their pedigree known. After considering the age of each male, and evaluating the founder lines represented by each male, one male in particular stands out as being the most important to the breeding colonies. Male #75 has a low inbreeding coefficient, and is from male founder line #7 and female line #12. Although founder lines #7 and #12 are adequately represented, the remaining males are from over-represented founder lines. As a result, male #75 would be a good candidate for reintroduction into a breeding colony.

8. Lastly, many of the individuals with the lowest mean kinship values are in the Seattle subpopulation, and these would therefore be valuable introductions to other breeding colonies.

Future Work

The determination of parentage using genetic analysis for the captive population of *E. helvum* has brought to light two interesting topics for behavioral research.

1. The assignment of maternity using behavioral observations agreed with molecular determination of parentage 79% of the time. A behavioral study to investigate the possibility that female *E. helvum* are performing complex behaviors such as alloparenting would be important.
2. Genetic and demographic analyses have shown an age specific peak in reproduction for male *E. helvum*. It would be interesting to investigate whether there is a behavioral component to this peak in male reproduction between the ages of 10 and 15.

Conclusions

As the first genetic study for this species, this research was able to determine the mating system in captivity, identify reproductive factors important to the management of the species, make specific population management recommendations to achieve parity of contribution between founding lines, and describe strategies used by *E. helvum* to maintain genetic diversity in a small isolated population. Future investigations of parentage and population structure in *E. helvum* would benefit from additional variable microsatellite loci. New variable loci would resolve parentage for the remaining cases where dams or sires are unknown, and could be used to clarify parentage in the cases where mitochondrial or microsatellite mutations have occurred.

The knowledge gained through this study of captive *E. helvum* can be used to study wild populations of *E. helvum*. Additionally, the molecular tools used in this study could be applied to other captive populations. The successful strategies

observed in the captive population of *E. helvum* can be used as a model for the management of small populations, both captive and wild.

Specific Hypotheses

There will be a decrease in heterozygosity and allelic diversity since founding in the captive population of E. helvum.

The hypothesis that heterozygosity and allelic diversity, as measured by microsatellite analysis, would decrease was not supported in the captive population of *E. helvum*. In fact, there was an overall increase in heterozygosity and allelic diversity. Because most founders have had multiple offspring, and many continue to have offspring, the chance of alleles being retained is high. Overlapping generations and a promiscuous mating system have allowed reproductively capable individuals to produce multiple offspring with low mean kinship values. As a consequence, both heterozygosity and allelic diversity have been maintained.

There will be an increase in inbreeding coefficients and mean kinship over time in the captive populations.

The hypothesis that there would be an increase in inbreeding coefficients and mean kinship values over time was not strongly supported in *E. helvum*. Over the past twenty years, the captive population of *E. helvum* has maintained relatively low inbreeding coefficients and mean kinship values. The majority of individuals have

inbreeding coefficients of zero. However, the individuals with unknown parentage may have had a substantial effect on the calculation of inbreeding coefficients and mean kinship. Promiscuous mating, continued founder contribution, and occasional migration seem to have slowed inbreeding and the accumulation of highly related individuals.

The mating system will be characterized by promiscuous mating.

The mating system for captive *E. helvum* is characterized as being promiscuous, as hypothesized. All of the analyses, including pedigree analysis, F-statistics, and inbreeding coefficient distributions, support this conclusion. A promiscuous mating system has contributed to the population's avoidance of the potential genetic and demographic problems associated with small populations.

There will be genetic differentiation over time between subpopulations over time as described by F-statistics.

The captive subpopulations of *E. helvum* have experienced only minor genetic differentiation, which does not support to the hypothesis that there would be differentiation between subpopulations over time. This lack of genetic differentiation has several contributing factors. Firstly, the founders are still present and continue to produce offspring. Secondly, substantial transferring of individuals between breeding colonies has occurred, with existing colonies seeding newer colonies with a relatively even representation of founder alleles.

There will be unequal founder contributions to the current population.

Unequal founder contribution was observed in the current captive *E. helvum* population, supporting the original hypothesis. This unequal founder contribution has several etiologies. First, male founder #10 was present in the population for only two years and was not part of the current study. Therefore, there are no identified offspring for this founder. Second, male founder #32 has had less success reproducing than has other males, and is known to have produced only one known offspring in eight years. Genetic testing has allowed the discovery of this deficiency, which may now be addressed and steps taken to correct it.

Summary

The captive population of *E. helvum* is well suited for captivity. They have been successfully breeding in captivity, and have displayed reproductive strategies that minimize the genetic and demographic obstacles associated with small populations. A promiscuous mating system, rapid population growth, overlapping generations, and long-term, near-equivalent founder contributions have proven to be a highly successful in maintaining genetic diversity and demographic stability in this small population. The reproductive strategy of the captive population of *E. helvum* is an excellent model for application to other small captive populations.

Table 5-1 Summary of Recommended Transfers of Individuals Between Institutions

	Oregon Zoo, Portland, OR	Woodland Park Zoo, Seattle, WA	Lincoln Park Zoo, Chicago, IL	Lubee Foundation Gainsville, FL	San Antonio Zoo, San Antonio, TX
Oregon Zoo, Portland, OR		A male and female from female founder-line #6; one offspring from male founder #1			
Milwaukee Zoo, Milwaukee, WI			#98; #75 or to Lubee		#116 or to Lubee
Woodland Park Zoo, Seattle, WA	A male and female from female founder-line #20				
Lubbe Foundation, Gainsville, FL	Consider sending a female from founder-line #2		#75 or to Milwaukee		#116 or to Milwaukee

Individuals are transferred from the institutions listed in the column heading, to the institutions in the row headings.

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Appendix A Genotypic and Parentage Data for All Individuals

Studbook #	Location	Local ID	Sex	[illegible]	[illegible]	[illegible]	[illegible]	Sire	Dam
1	Seattle	21014	M	BD	AC	BB	VII	Wild	Wild
5	Milwaukee	2625	F	CD	CC	DD	II	Wild	Wild
6	Milwaukee	2624	F	CC	AC	AB	VI	Wild	Wild
7	Milwaukee	2620	M	AD	1C	BC	VII	Wild	Wild
8	Milwaukee	2621	M	AD	BC	BB	IV	Wild	Wild
12	Milwaukee	2622	F	DD	BC	BB	IV	Wild	Wild
13	Milwaukee	2623	F	CD	CC	DD	II	Wild	Wild
16	Milwaukee	2719	M	AD	BC	BC	IV		2622
20	Portland	90115	F	CD	BC	AC	V	Wild	Wild
23	Portland	91051	F	DD	CC	BC	III		2623
25	ChicagoLP	10056	M	CD	CC	AB	VI	2621	2624
32	Portland	90114	M	BD	AE	BD	VIII	Wild	Wild
33	Seattle	890290	F		AD	BC	VI		2624
35	Franklin Park	90A797	M	CD	CC	BC	III		2623
37	Franklin Park	90A798	F	AC	AC	AC	VI	2620	2624
42	Franklin Park	90A792	M	DD	CC	AB	IX		21015
43	Milwaukee	3360	F	DD	BD	BC	IV		2622
44	ChicagoLP	10057	M	CD	BC	AD	V		90115
45	Portland	91052	F	CD	CC	BB	III	2621	2623
46	Portland	91050	M	DD	CC	DD	II		2625
47	Seattle	891097	F	BD	AB	CC	VI		890290
48	Portland	91053	F	DD	CD	BB	III		91051
49	Portland	91048	M	DD	BC	BC	IV	90A797	2622
50	Portland	91300	M	CD	1C	CD	I	21014	21015
52	Portland	91054	F	AC	BC	CD	II	2620	2625
53	ChicagoLP	10022	M	DD	BC	BC	IV	90A797	2622
56	Portland	91049		DD	CC		III	90A797	2623
57	Portland	91024	F	CD	AC	BB	III		
59	Portland	93102	F	BD	CC	AD	IX		21015
62	Milwaukee	3599	F	BC	CD	CD	II		2625
64	Milwaukee	3608	M	CC	CC	BD	III	90A797	2623
65	Portland	91274	M		AC	AB	III		91051
66	Portland	91301	M	DD	CC	BB	III	91048	91052
67	Milwaukee	3614	F	AD	CD	BC	IV	2620	3360
68	Portland	91305	M	CD	FF	CC	V	91048	90115
70	Portland	92004	M	DD	CC	CC	VI	91048	
71	Milwaukee	3725	F	CD	BC	BC	IV		
72	Milwaukee	3735	F	AD	BD		IV	2621	
73	ChicagoLP	10023	M	AC	1A	BC	III	2620	2623
74	ChicagoLP	10024	M	CD	BC	BD	II	2621	2625
75	ChicagoLP	10025	M	AD	BC	BC	IV	2620	2622
80	ChicagoLP	10058	M	DD	CD		III		91024
81	Portland	93004	M	CD	CD	AB	III	10057	91053
89	Milwaukee	3872	F	AB	CC	BC	II	2621	3599
91	Franklin Park	94A086	F	AD	BC	BC	IV	2620	2622
92	Franklin Park	94A087	F	DD	BD	BC	IV	2621	3360

Appendix A Genotypic and Parentage Data for All Individuals

Studbook #	Location	Local ID	Sex	[illegible]	[illegible]	[illegible]	[illegible]	Sire	Dam
93	Milwaukee	3895	M	AD	BC	BD	II	2621	2625
96	Portland	94014	F	CD	CC	CD	II	92004	91054
97	Portland	94015	F	CD	BC	AC	V	92004	90115
98	ChicagoLP	10029	M	DD	BC	BC	VI	2621	
99	ChicagoLP	10026	M	AD	BD	BB	IV	2621	3735
100	Seattle	940306	M	DD	DE		III	91048	91053
101	Portland	94018	F	CD	DD		V	91305	
103	Portland	94126	F	DD	CC	BB	IX	91301	93102
104	ChicagoLP	10028	M	CD	BC	AB	VI	2621	2624
105	Milwaukee	3985	F	DD	CC	BC	VI	2620	
106	Milwaukee	4009	F	CD		BD	II	2621	3599
107	Milwaukee	3993	F	AD	BD	BB	IV	2621	3360
109	Milwaukee	4010	M	AA	BC	BD	IV	2621	3735
111	Milwaukee	4003	F	CD	AC	AB	III	10023	2623
112	Milwaukee	4011	F	DD	CC	CD	VI	10024	
113	Portland	95002	M	AD	DD	CC	II	92004	91054
114	Portland	95003	M	CD	AB	AB	V	91274	90115
116	ChicagoLP	10059	M	DD	CC	AB	IX	91301	93102
117	Portland	95001	M	DD	CC	BB	III	91301	91051
119	Portland	95013	M	DD	1C		III	91300	91053
120	Seattle	950022	M	BD	AC	BB	VI		890290
123	Seattle	950362	M	DD	AA	BC	VI		891097
124	Milwaukee	4125	F	AB	1D	BC	II	2620	3599
126	Milwaukee	4128	F	CD	BC	BC	IV		2622
130	ChicagoLP	10027	M	CC	CC	DD	II	3608	2625
131	Milwaukee	4179	M	CD	BC	CD	IV	10024	?
134	Milwaukee	4143	M	DD	CC	BC	VI		3985
141	Portland	96006	F	CC	BC	BC	III	91305	91052
143	Milwaukee	4184	F	AC	CD	BB	IV	10026	?
147	ChicagoLP	10060	M	CD	BC	CD	II	92004	91054
148	ChicagoLP	10061	M	CD	AC	BB	III	91274	91053
149	Portland	96012	M	DD	BC		III	91048	91024
150	ChicagoLP	10062	M	CD	CC	BC	V	91301	94015
152	Franklin Park	96F247	?	DD	AC	AB	VI	90A792	94F127
154	Seattle	960191	F	BD	DD	BC	VI		890290
158	Milwaukee	4242	M	DD	AB	BB	III		4003
160	Milwaukee	4250	?	AD	BC	BC	IV	10025	
165	Milwaukee	4284	?	DD	CC	BB	III		2623
166	Milwaukee	4285	?	AA	AB	BB	VI	2621	2624
168	Milwaukee	4287	?	CD	CD	CD	IV	3608	3360
169	Milwaukee	4288	M	CD	BC	CC	II		3599
171	Milwaukee	4290	?	AB	BC	CC	II		3599
172	Milwaukee	4291	M	DD	BC	BC	IV		3993
174	Milwaukee	4293	?	CC	BC	BC	II		4009
175	Milwaukee	4341	M	CD	1B	CD	II	10024	2625
176	Milwaukee	4342	M	CC	BC	BC	IV	3608	3725

Appendix A Genotypic and Parentage Data for All Individuals

Studbook #	Location	Locality	Sex	P1	P2	Alleles	PHP	Size	Count
178	Portland	97000	M	BC	CC	AB	IX	10056	93102
179	Portland	97001	F	CD	AB	AB	V	91274	90115
184	Portland	97005	F				II		
185	Portland	97006	M	CD	BC		II	91048	91054
186	Portland	97007	M	CD	DD		V	93004	94018
213	Milwaukee	4376		BB	CC	BC	II		3599
214	Milwaukee	4384		DD	BD		IV		
215	Milwaukee	4441		CD		AB	VI		3985
216	Milwaukee	4442		CD		BB	X	3608	
257	Milwaukee	4110				CD	II		
258	Milwaukee	4375	F	DD	AC	BD	III	2621	2623
261	Milwaukee	4385		AD	CC	CD	II	3895	3872
263	Milwaukee	4440				BC	IV	2620	
264	Milwaukee	4443		CD		BD	II	2621	3599
266	Milwaukee	4445		DD	BC	BD	II	3895	4009
267	Milwaukee	4446		CC	CC		X	3608	
268	Milwaukee	4447		AD	BC	BD	IV	3895	
269	Milwaukee	4456		CD	BC	BB	IV		
270	Milwaukee	4457		AC	CD	BD	II	4287	3872
271	Milwaukee	4458		AD	BC	BB	IV	2621	
272	Milwaukee	4459		CD		BD	IV	3608	
273	Milwaukee	4460		CD	AB	BD	III	3895	4003
274	Milwaukee	4461		DD	CC	BD	II	2621	2625
275	Milwaukee	4462		CC	BC	CC	IV		
276	Milwaukee	4463		AD	CC	BC	IV	2620	
	Milwaukee	?		DD	BC	BC	IV		
	Milwaukee	00-0021-		CC	CC	BD	II		
	Milwaukee	4482		AA	AB	BC	VI		2624

Appendix B **Dams and Pups**

Dam	Pup	Year	Locus 4	Locus 9	Locus 18	mtDNA
101			**CD**	**DD**		**V**
	186	1997	CD	DD	V	
105			**DD**	**CC**	**BC**	**VI**
	134	1996	DD	CC	BC	VI
	215	1998	CD	CC	AB	VI
106			**CD**		**BD**	**II**
	160	1996	**AD**	BC	**BC**	II
	266	1998	DD	BC	**BD**	II
	175	1997	CD	1B	CD	II
107			**AD**	**BD**	**BB**	**IV**
	172	1997	DD	BC	BC	IV
	271	1998	**AD**	**BC**	BB	IV
12			**DD**	**BC**	**BB**	**IV**
	16	1985	**AD**	**BC**	**BC**	IV
	43	1989	DD	BD	BC	IV
	49	1990	**DD**	**BC**	**BC**	IV
	53	1990	**DD**	**BC**	**BC**	IV
	75	1992	**AD**	**BC**	**BD**	IV
	110	1994				
	126	1995	**CD**	BC	**BC**	IV
	91	1993	**AD**	**BC**	**BC**	IV
13			**CD**	**CC**	**DD**	**II**
	23	1986	DD	CC	BC	III
	39	1988				
	35	1988	CD	CC	BC	III
	56	1997	**DD**	**CC**		III
	64	1991	**CC**	**CC**	**BD**	III
	73	1992	**AC**	**IA**	**BC**	III
	111	1994	CD	**AC**	**AB**	III
	45	1989	**CD**	CC	BB	III
	165	1997	DD	CC	BB	III

Appendix B **Dams and Pups**

Dam	Pup	Year	Locus 4	Locus 9	Locus 18	mtDNA
111			**CD**	**AC**	**AB**	**III**
	158	1996	**D**D	A**B**	**B**B	III
	275	1998	C**D**	A**B**	**B**D	III
	258	1997	**DD**	A**C**	**B**D	III
2						
	42	1989	DD	CC	AB	IX
	50	1990	CD	IC	CD	I
	59	1991	BD	CC		IX
20			**CD**	**BC**	**AC**	**V**
	97	1994	C**D**	**B**C	A**C**	V
	114	1995	C**D**	A**B**	**AB**	V
	179	1997	C**D**	A**B**	**AB**	V
	68		**CD**	FF	**CC**	V
23			**DD**	**CC**	**BC**	**III**
	117	1995	DD	CC	BB	III
	48	1990	DD	CD	BB	III
	65	1991		A**C**	A**B**	III
33				**AD**	**BC**	**VI**
	47	1989	BD	A**B**	**C**C	VI
	120	1995	BD	A**C**	BC	VI
	154	1996	BD	**DD**	BC	VI
47			**BD**	**AB**	**CC**	**VI**
	123	1995	**DD**	AA	BC	VI
43			**DD**	**BD**	**BC**	**IV**
	168	1997	C**D**	C**D**	**CD**	IV
	67	1991	A**D**	C**D**	BC	IV
	107	1994	A**D**	B**D**	**BB**	IV
	92	1993	**DD**	B**D**	BC	IV

Appendix B **Dams and Pups**

Dam	Pup	Year	Locus 4	Locus 9	Locus 18	mtDNA
45			**CD**	**CC**	**BB**	**III**
	66	1991	**D**D	**C**C	**B**B	III
	141	1996	**C**C	B**C**	**B**C	III
48			**DD**	**CD**	**BB**	**III**
	81	1993	C**D**	C**D**	A**B**	III
	100	1994	**D**D	**D**E		III
	119	1995	**D**D	**I**C		III
	148	1996	C**D**	A**C**	B**B**	III
5			**CD**	**CC**	**DD**	**II**
	52	1990	A**C**	B**C**	C**D**	II
	62	1991	B**C**	C**D**	C**D**	II
	93	1993	A**D**	B**C**	B**D**	II
	46	1989	**D**D	**C**C	**D**D	II
	74	1992	C**D**	B**C**	B**D**	II
	130	1996	**C**C	**C**C	**D**D	II
	274	1998	**D**D	**C**C	B**D**	II
52			**AC**	**BC**	**CD**	**II**
	96	1994	**C**D	**C**C	C**D**	II
	147	1996	**C**D	**B**C	C**D**	II
	185	1997	**C**D	**B**C		II
57			**CD**	**AC**	**BB**	**III**
	80	1993	**D**D	C**D**		III
	149	1996	**D**D	B**C**		III
59			**BD**	**CC**		**IX**
	116	1995	**D**D	**C**C	**A**B	IX
	103	1994	**D**D	**C**C	**B**B	IX
	178	1997	**B**C	**C**C	AB	IX

Appendix B **Dams and Pups**

Dam	Pup	Year	Locus 4	Locus 9	Locus 18	mtDNA
62			**BC**	**CD**	**CD**	**II**
	124	1995	A**B**	1**D**	BC	II
	169	1997	CD	BC	CC	II
	89	1993	A**B**	**CC**	**BC**	II
	213	1998	BB	CC	BC	II
	264	1998	**CD**	CC	BD	II
	171	1997	A**B**	B**C**	**CC**	II
	106	1994	**CD**		**BD**	II
71			**CD**	**BC**	**BC**	**IV**
	176	1997	**CC**	**BC**	**BC**	IV
72			**AD**	**BD**		**IV**
	99	1994	AD	B**D**	BB	IV
	109	1994	**AA**	**BC**	B**D**	IV
89			**AB**	**CC**	**BC**	**II**
	261	1998	**AD**	**CC**	**CD**	II
	270	1998	AC	CD	BD	II
6			**CC**	**AC**	**AB**	**VI**
	25	1986	**CD**	**CC**	**AB**	VI
	37	1988	A**C**	**AC**	**AC**	VI
	104	1994	**CD**	**BC**	**AB**	VI
	33	1988		AD	BC	VI
	166	1997	AA	**AB**	**BB**	VI
	4482	1998	AA	**AB**	**BC**	VI
97			**CD**	**BC**	**AC**	**V**
	150	1996	**CD**	**CC**	**BC**	V
50						
	113	1995	AD	DD	CC	II

Legend Indicates mis-match , Bold type = allele given by dam

Appendix C **Sires and Pups**

Sire	Pup	Year	Locus 4	Locus 9	Locus 18	Conf. Limit
	8		**AD**	**BC**	**BB**	
	25	1986	**CD**	CC	**AB**	(-)
	45	1989	**CD**	CC	**BB**	(-)
	72	1992	AD	**BD**		(-)
	74	1992	**CD**	**BC**	**BD**	(-)
	89	1993	**AB**	**CC**	**BC**	(-)
	92	1993	**DD**	**BD**	**BC**	(-)
	93	1993	**AD**	**BC**	**BD**	*
	98	1994	**DD**	BC	**BC**	(-)
	99	1994	AD	**BD**	BB	*
	104	1994	**CD**	**BC**	**AB**	(-)
	106	1994	**CD**		**BD**	(-)
	107	1994	**AD**	**BD**	**BB**	(+)
	109	1994	**AA**	**BC**	**BD**	(-)
	166	1997	**AA**	**AB**	**BB**	(-)
	274	1998	**DD**	**CC**	**BD**	(-)
	271	1998	**AD**	BC	BB	(-)
	264	1998	**CD**	CC	**BD**	(-)
	7		**AD**	**1C**	**BC**	
	37	1988	**AC**	**AC**	**AC**	*
	73	1992	**AC**	**1A**	**BC**	(-)
	75	1992	**AD**	**BC**	**BC**	*
	91	1993	**AD**	**BC**	**BC**	*
	105	1994	**DD**	CC	BC	(-)
	124	1995	**AB**	**1D**	**BC**	*
	263	1998			BC	(-)
	276	1998	AD	CC	BC	(-)
	175	1997	**CD**	**1B**	**CD**	(-)
	16	1985	**AD**	**BC**	**BC**	
	67	1991	**AD**	**CD**	BC	(-)

Appendix C **Sires and Pups**

Sire	Pup	Year	Locus 4	Locus 9	Locus 18	Conf. Limit
	1		**BD**	**AC**	**BB**	
		3 1980				
		4 1981				
		14 1983				
		15 1984				
		4 1985				
		24 1986				
		29 1987				
		36 1988				
		42 1989	**DD**	CC	**AB**	*
		77 1992				
		102 1994				
	3					
		59 1993	**BD**	CC	AD	
		50 1990	**CD**	1**C**	CD	
	44		**CD**	**BC**	**AD**	
		81 1993	**CD**	**CD**	**AB**	(-)
	74		**CD**	**BC**	**BD**	
		112 1994	**DD**	**CC**	**CD**	(-)
		131 1996	**CD**	BC	**CD**	(-)
	93		**AD**	**BC**	**BD**	
		261 1998	**AD**	CC	**CD**	(+)
		266 1998	**DD**	**BC**	BD	(-)
		268 1998	AD	BC	BD	(-)
		273 1998	**CD**	**AB**	**BD**	(-)
		258 1997	**DD**	AC	**BD**	
	42		**DD**	**CC**	**AB**	
		144 1996				
		145 1996				
		152 1996	DD	AC	AB	(+)

Appendix C **Sires and Pups**

Sire	Pup	Year	Locus 4	Locus 9	Locus 18	Conf. Limit
50			**CD**	**1C**	**CD**	
	119	1995	**D**D	**1**C		*
65				**AC**	**AB**	
	114	1995	C**D**	**A**B	A**B**	*
	148	1996	C**D**	**A**C	**B**B	*
	179	1997	C**D**	**A**B	A**B**	*
35			**CD**	**CC**	**BC**	
	49	1990	**DD**	**B**C	B**C**	(-)
	53	1990	**DD**	**B**C	B**C**	(-)
	56	1991	**DD**	**C**C		(-)
	64	1991	**C**C	**C**C	**B**D	(+)
	108	1994				
49			**DD**	**BC**	**BC**	
	66	1991	**DD**	**C**C	**B**B	(+)
	68	1991	**CD**	FF	**C**C	(-)
	70	1992	DD	CC	CC	(-)
	149	1996	**DD**	**B**C		*
	185	1997	**CD**	BC		(-)
66			**DD**	**CC**	**BB**	
	103	1994	**DD**	**C**C	**B**B	(+)
	116	1995	**DD**	**C**C	A**B**	(+)
	117	1995	DD	CC	BB	(+)
	150	1996	**CD**	**C**C	**B**C	(+)
70			**DD**	**CC**	**CC**	
	96	1994	**CD**	**C**C	**C**D	(-)
	97	1995	**CD**	**B**C	A**C**	(-)
	147	1996	**CD**	**B**C	**C**D	(-)

Appendix C **Sires and Pups**

Sire	Pup	Year	Locus 4	Locus 9	Locus 18	Conf. Limit
64			**CC**	**CC**	**BD**	
	130	1996	CC	CC	DD	*
	168	1997	**CD**	**CD**	**CD**	*
	176	1997	**CC**	**BC**	**BC**	(-)
	216	1998	**CD**		**BB**	(-)
	267	1998	CC	CC		(+)
	272	1998	**CD**		BD	(-)
82						
	193	1997				
25			**CD**	**CC**	**AB**	
	178	1997	**BC**	CC	AB	(+)
75			**AD**	**BC**	**BC**	
	160	1996	**AD**	**BC**	BC	(_)
32			**BD**	**AE**	**BD**	
	100	1998	**DD**	DE		(-)
16			**AD**	**BC**	**BC**	
	52	1990	**AC**	**BC**	**CD**	(-)
19						
	57	1991		**AC**	**AB**	

Legend (*) = 90%, (+) = 80%, (-) = ,80%
Bold type = allele given by sire

Appendix D Descendant List For Female Founder #12

12						F			
	16					M	7		
		52				F	5		
			76			M	UNK		
			96			F	70		
				184		F	UNK		
					227	U	UNK		
			147			M	70		
			185			M	49		
			217			F	UNK		
			224			M	UNK		
	19					M	UNK		
	28					M	UNK		
	43					F	UNK		
		58				F	UNK		
		67				F	UNK		
		92				F	8		
			144			U	42		
			196			U	UNK		
		107				F	8		
			172			M	UNK		
		168				U	64		
	49					M	35		
		66				M	45		
			103			F	59		
			140			M	UNK		
			116			M	59		
			117			M	45		
			150			M	97		
		68				M	20		
		70				M	UNK		
			96			F	52		
				184		F	UNK		
					227	U	UNK		
			97			F	20		
				150		M	66		
				218		F	UNK		

Appendix D Descendant List For Female Founder #12

			147			M	52		
		149				M	57		
		185				M	52		
	53					M	35		
	75					M	7		
		160				F	106		
	91					F	7		
	110					F	UNK		
	126					F	UNK		
	164					F	UNK		
Legend: Bats indicated by gray shading were not included in this study.									
Offspring are placed below and one column to the right of the parent. All									
individuals in the same column are siblings.									

Appendix D Descendant List For Female Founder #9

9				F				
	21			U	UNK			
	25			M	8			
		178		M	59			
	33			F	UNK			
		47		F	UNK			
			102	U				
			121	U	UNK			
			123	U	UNK			
			157	U	UNK			
			194	U	UNK			
		120		U	UNK			
		154		F	UNK			
		195		U	UNK			
	37			F	7			
		54		U	UNK			
		61		U	UNK			
		69		U	UNK			
		84		F	UNK			
		97		U	UNK			
		108		F	35			
			146	U	UNK			
			189	U	UNK			
		122		M	UNK			
		145		U	42			
		152		U	42			
		156		U	UNK			
	51			F	UNK			
	104			M	8			
	166			U	8			
	4482			U	171			

Legend: Bats indicated by gray shading were not included in this study. Offspring are placed below and one column to the right of the parent. All individuals in the same column are siblings.

Appendix D Descendant List For Femal Founderr #20

20				F				
	44			M	UNK			
		81		M	48			
			186	M	101			
	68			M	49			
	79			F	UNK			
		113		M	UNK			
		128		F	UNK			
		159		M	UNK			
	97			F	70			
		150		M	66			
		218		F	UNK			
	114			M	65			
	137			M	UNK			
	179			F	65			
	198			M	UNK			
	223			M	UNK			
Legend: Bats indicated by gray shading were not included in this study.								
Offspring are placed below and one column to the right of the parent. All								
individuals in the same column are siblings.								

Appendix D Descendant List For Female Founder #2

2				F				
	3			M	1			
	4			M	1			
	14			U	1			
	15			U	1			
	18			M	1			
	24			U	1			
	29			F	1			
		85		U	U			
	36			M	1			
	42			M	1			
		144		U	92			
		145		U	37			
		152		U	37			
	50			M	1			
		119		M	48			
	59			F	1			
		103		F	66			
			140	M	U			
		116		M	66			
		153		U	U			
		178		M	25			
	77			M	1			

Legend: Bats indicated with gray shading were not included in this study.

Offspring are placed below and one column to the right of the parent. All individuals in the same column are siblings.

Appendix D Descendant List for Female Founder #5

5					F			
	22				M	UNK		
	40				F	UNK		
	46				M	UNK		
	62				F	UNK		
		83			F	UNK		
			155		U	UNK		
		89			F	8		
			261		U	93		
			270		U	UNK		
		106			F	8		
			160		F	75		
			175		M	7		
			266		U	93		
		124			F	7		
		169			M	UNK		
		171			U	UNK		
			4482		U	6		
		213			F	UNK		
		264			F	8		
	74				M	8		
		112			F			
		131			M	UNK		
	93				M	8		
		261			U	89		
		266			U	106		
		268			U	UNK		
		275			M	111		
		258			F	111		
	130				M	64		
	167				U	UNK		
	274				U	8		
	52				F	16		
		76			M	UNK		
		96			F	70		
			184		F	UNK		
				227	U	UNK		

Appendix D Descendant List for Female Founder #5

.		147			M	70		
		185			M	49		
		217			F	UNK		
		224			M	UNK		
Legend: Bats indicated with gray shading were not included inthis study.								
Offspring are placed below and one column to the right of the parent. All								
individuals in the same column are siblings.								

Appendix D Descendant List For Female Founder #13

13							F		
	23						F	UNK	
		48					F	UNK	
			81				M	44	
				186			M	101	
			100				M	32	
			119				M	50	
			148				M	65	
			187				F	UNK	
			219				M	UNK	
			226				U	UNK	
		65					M	UNK	
			114				M	20	
			148				M	48	
			179				F	20	
		78					M	UNK	
		94					M	UNK	
		125					M	UNK	
		183					M	UNK	
		201					M	UNK	
		225					M	UNK	
	35						M	UNK	
		49					M	12	
			66				M	45	
				103			F	59	
					140		M	UNK	
				116			M	59	
				117			M	45	
				150			M	97	
			68				M	20	
			70				M	UNK	
				96			F	52	
					184		F	UNK	
						227	U	UNK	
				97			F	20	
					150		M	66	
					218		F	UNK	

Appendix D Descendant List For Female Founder #13

				147			M	52	
			149				M	57	
			185				M	52	
		53					M	12	
		56					M	13	
		64					M	13	
			130				M	5	
			168				U	43	
			176				M	71	
			216				M	UNK	
			267				U	UNK	
			272				U	UNK	
		108					F	37	
			146				U	UNK	
			189				U	UNK	
	39						M	UNK	
	45						F	8	
		66					M	45	
			103				F	59	
				140			M	UNK	
			116				M	59	
			117				M	45	
			150				M	97	
		182					M	UNK	
			193				M	143	
		117					M	66	
		141					F	UNK	
			220				F	UNK	
		181					M	UNK	
		182					M	UNK	
		200					M	UNK	
		222					M	UNK	
	56						M	35	
	64						M	35	
		130					M	5	
		168					U	43	
		176					M	71	

Appendix D Descendant List For Female Founder #13

		216					M	UNK	
		267					U	UNK	
		272					U	UNK	
	73						M	7	
	111						F		
		158					M	UNK	
		275					M	93	
		258					F	93	
	165						U	UNK	
Legend: Bats indicated with gray shading were not included in the study.									
Offspring are placed below and one column to the right of the parent. All									
individuals in the same column are siblings.									

Appendix E Inbreeding Coefficients and Mean Kinship Values for All Individuals

Studbook	F	Mean Kinship	Studbook	F	Mean Kinship
#1	0	0.0407	#56	0.5	0.1228
#4	0	0.0423	#57		0
#5	0	0.0706	#58	0	0.0644
#6	0	0.0494	#59	0	0.0469
#7	0	0.0554	#60	0	0.0737
#8	0	0.0549	#62	0	0.0919
#10	0	0	#63		0
#12	0	0.0523	#64	0.5	0.1244
#13	0	0.1088	#65	0	0.1435
#14	0	0.0423	#66	0.1667	0.106
#16	0	0.0554	#68	0	0.0758
#18	0	0.0423	#70	0	0.1268
#20	0	0.0302	#71		0
#21	0	0.0525	#72	0	0.0913
#22	0	0.0737	#73	0	0.0907
#23	0	0.1435	#74	0	0.0689
#25	0	0.0568	#75	0	0.0601
#28	0	0.0554	#76	0	0.0812
#29	0	0.0423	#77	0	0.0423
#30	0	0.0525	#78	0	0.01435
#31		0	#79	0	0.0333
#32	0	0	#80		0
#33	0	0.0618	#81	0	0.0716
#34		0	#82	0	0.1435
#35	0	0.1324	#83	0	0.0919
#37	0	0.0625	#86		0
#39	0	0.1119	#88		0
#42	0	0.0438	#89	0	0.0919
#43	0	0.0644	#90		0
#44	0	0.0357	#91	0	0.057
#45	0	0.1435	#92	0	0.0611
#46	0	0.0737	#93	0	0.0689
#47	0	0.0618	#94	0	0.1435
#48	0	0.1435	#95		0
#49	0	0.0873	#96	0	0.0953
#50	0	0.0488	#97	0	0.0611
#51	0	0.0525	#98	0	0.0632
#52	0	0.0781	#99	0.125	0.0785
#53	0	0.0811	#100	0.1667	0.1029

Appendix E Inbreeding Coefficients and Mean Kinship Values for All Individuals

Studbook	F	Mean Kinship	Studbook	F	Mean Kinship
#101	0	0.0796	#147	0	0.0936
#102	0	0.0474	#148	1	0.1435
#103	0	0.069	#149	0	0.0894
#104	0	0.0552	#150	0.0796	0.0814
#105	0	0.0616	#151		0
#106	0	0.0713	#152	0	0.0563
#107	0	0.0616	#154	0	0.0618
#108	0	0.0889	#156	0	0.0656
#109	0.125	0.0762	#158	0	0.1129
#110	0	0.0554	#160	0	0.0632
#111	0.25	0.1106	#163		0
#112	0	0.5	#164	0	0.0554
#113	0	0.6875	#165	0	0.1176
#114	0	0.625	#166	0	0.0589
#115		0	#167	0	0.0737
#116	0	0.069	#168	0	0.1013
#117	0.375	0.1192	#169	0	0.0919
#118		0	#170		0
#119	0	0.0703	#171	0	0.0919
#120	0	0.0618	#172	0	0.0647
#122	0	0.0656	#174	0	0.0734
#123	0	0.0618	#175	0	0.0737
#124	0	0.0697	#176	0	0.1255
#125	0	0.1435	#177		0
#126	0	0.0554	#178	0	0.055
#127		0	#179	0	0.0554
#129		0	#183	0	0.1435
#130	0	0.0959	#184	0	0.0979
#131	0	0.072	#185	0	0.0847
#132		0	#186	0.0666	0.0758
#133		0	#187	0	0.1435
#134	0	0.0616	#188		0
#135	0	0.0796	#193	0.125	0.0948
#137	0	0.0333	#194	0	0.0618
#138		0	#195	0	0.0618
#141	0.2	0.0965	#196	0	0.0632
#142		0	#198	0	0.0333
#143	0	0.0826	#199	0	0.0796
#146	0	0.091	#200	0	0.1435

Appendix E Inbreeding Coefficients and Mean Kinship Values for All Individuals

Studbook	F	Mean Kinship	Studbook	F	Mean Kinship
#201	0	0.1435	#269		0
#202		0	#270	0	0.1
#204		0	#271	0	0.058
#205		0	#272	0	0.1255
#206		0	#275	0	0.1106
#207		0	#274	0	0.0883
#208		0	#275	0	0.0919
#209		0	#276	0	0.0585
#210		0	4482	0	0.0569
#212		0			
#213	1	0.0919			
#214	0	0.0647			
#215		0			
#216		0			
#217	0	0.0812			
#218	0	0.0628			
#219	0	0.1435			
#220	0	0.0974			
#221	0	0.0796			
#222	0	0.1435			
#223	0	0.0333			
#224	0	0.0812			
#225	0	0.1435			
#226	0	0.1435			
#227	0	0.0979			
#228		0			
#229		0			
#230		0			
#231		0			
#232		0			
#233		0			
#261	0.25	0.076			
#263		0			
#215	0	0.0616			
#216	0	0.1047			
#264	0	0.1047			
#266	0	0.0911			
#267	0	0.1255			
#268	0	0.072			