POPULATION GENETICS OF THE ENDANGERED MIAMI BLUE BUTTERFLY *Cyclargus thomasi bethunebakeri*: IMPLICATIONS FOR CONSERVATION

By

EMILY VIRGINIA SAARINEN

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

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By

Emily Virginia Saarinen

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Chair: Jaret C. Daniels
Co-chair: Jacqueline Y. Miller
Major: Entomology and Nematology

The Miami blue butterfly (*Cyclargus thomasi bethunebakeri*) (Lepidoptera: Lycaenidae) is endemic to the state of Florida and, until the 1980s, was locally common throughout much of the southern mainland, western barrier islands and the Florida Keys. Over the last few decades, the butterfly's overall distribution and numerical abundance have been significantly reduced and a variety of factors have been identified as potential causes of overall decline. In 1999, the butterfly was rediscovered as part of a small breeding population in Bahia Honda State Park in the lower Florida Keys and in 2007 an additional population was found in Key West National Wildlife Refuge. A captive breeding colony from Bahia Honda State Park individuals was established in order to provide organisms for reintroduction and research. The current level of existing molecular diversity and gene flow of wild and captive populations are unknown, and I present data from twelve polymorphic microsatellite loci to assess the populations. Analysis of museum specimens shows that populations were historically widespread and genetically diverse. Some diversity has been lost in the current populations, however a greater genetic diversity (allelic diversity and heterozygosity) than originally predicted exists in both Bahia Honda State Park and Key West National Wildlife Refuge. Allelic frequencies also show short-range gene

flow between habitat patches within Bahia Honda State Park. I show that the captive colony maintains representative genetic diversity from the wild colony, indicating that captive-bred individuals are genetically suitable for reintroduction to new sites. Genetic distances are increased between the wild colony and the captive colony when new wild stock is not integrated to the captive colony on a regular basis. Laboratory experiments with different numbers of founding individuals emphasize the importance of how stochastic demographic factors negatively impact small populations. The ultimate purpose of this project is to gain critical scientific information on this state-endangered butterfly in order to facilitate informed decision making regarding the short and long-term management of the taxon so as to secure a stable or increasing population at a level that does not require protection.

CHAPTER 1
INTRODUCTION

History of the Miami Blue Butterfly

The Miami blue butterfly *Cyclargus thomasi bethunebakeri* (Comstock and Huntington) (Lepidoptera: Lycaenidae: Polyommatinae) is a small, brightly colored butterfly endemic to south Florida, with additional species occurring throughout the Caribbean basin (Smith et al. 1994). Historically, little information has been available on the complete extent of the taxon's range within Florida, but most authors agree that it once commonly occurred from the southern mainland through the Florida Keys and the Dry Tortugas (Klots 1964; Lenczewski 1980; Minno & Emmel 1993; Calhoun et al. 2002). Additional reports of *C. t. bethunebakeri* from the Bahamas, the Bimini Islands, are unverifiable as there are no specimens located in museum holdings (L.D. Miller, personal communication). These likely represent irregular, non-breeding strays (Calhoun et al. 2002). Primarily a coastal species, *C. t. bethunebakeri* inhabits tropical coastal hammocks and beachside scrub. Over the last few decades, the butterflies' overall distribution and numerical abundance have been significantly reduced (Minno & Emmel 1994; Calhoun et al. 2002). Rapid urbanization and the associated loss of coastal habitat have eliminated *C. t. bethunebakeri* from the south Florida mainland. The butterfly has become considerably rarer and was thought to have been extirpated with no verified records from March 1992 to October 1999 (Calhoun et al. 2002). The taxon was rediscovered in November 1999 as part of a small population within the boundaries of Bahia Honda State Park (BHSHP) on Bahia Honda Key in the lower Florida Keys (Ruffin & Glassberg 2000). Independent lepidopterists specializing in the taxonomy of this group have verified that the butterflies discovered on BHSP are indeed the same subspecies as historic specimens (L.D. and J.Y. Miller, personal communication). In December 2007, an additional population was discovered in Key West

National Wildlife Refuge (KWNWR) 50 km west of Key West, Florida (Cannon 2007). No taxonomic verification by genitalic dissection of these butterflies has occurred due to permit constraints of the KWNWR, but preliminary molecular evidence has confirmed that they are the same taxon (E.V. Saarinen, unpublished data).

History of the Genus *Cyclargus* Nabokov 1945

The genus *Cyclargus* was split from the genus *Hemiargus* Hübner 1885 by Nabokov in 1945 (Nabokov 1945). Nabokov made this distinction based on structural differences in the male genitalia between the two groups and designated *C. ammon* Lucas 1857 from Cuba as the type species for the genus (Nabokov 1945). In his description of the new genus *Cyclargus*, Nabokov designated the species *C. dominica* Möschler 1886 from Jamaica, *C. thomasi* Clench 1941 from Cat Island (Bahamas), and *C. woodruffi* Comstock & Huntington 1943 from Anegada (Virgin Islands) as members of the genus. Riley (1975) incorrectly re-synonomized the genera *Hemiargus* and *Cyclargus* despite Nabokov's earlier work. Riley apparently sank *Cyclargus* back in *Hemiargus* due to similar wing characteristics but failed to distinguish the genitalic distinctions recognized by Nabokov (Johnson & Bálint 1995). In their revision of neotropical blue butterflies (Polyommatinae), Johnson and Matusik (1988) discovered this taxonomic discrepancy and addressed the issue in later publications (Johnson & Bálint 1995). However, Riley's earlier generic distinctions are still perpetuated by many authors, including those of several recent field guides (Opler & Malikul 1992; Allen et al. 2005; Minno et al. 2005). Bálint and Johnson (1995) name eight species in *Cyclargus* (*ammon, erembis, thomasi, woodruffi, dominica, kathleena, sorpresus,* and *kathleena*). Treatment of the genus by Smith et al. (1994) includes a new species from Puerto Rico, bringing the total to nine species (Table 1-1).

The Miami blue butterfly *Cyclargus thomasi bethunebakeri* (Comstock and Huntington) is endemic to Florida with additional subspecies occurring in the Bahamas, Puerto Rico, and

Hispaniola (Smith et al. 1994) (Table 1-2). The type specimen of *C. t. bethunebakeri* is housed at the American Museum of Natural History (AMNH) and the type locale is Miami, Florida. This subspecies is often written as *bethune-bakeri,* but the original description does not hyphenate the subspecies name (Comstock & Huntington 1943).

Listing Status of *Cyclargus thomasi bethunebakeri*

The decline of *Cyclargus thomasi bethunebakeri* may have begun gradually, but it has become dramatic within recent years. This butterfly is currently one of the most endangered taxa in the world due to its low population numbers and presence at a fraction of its original geographic range. Through the joint efforts of both state and federal agencies, it was given emergency status as a state-endangered taxon in November 2002 by the Florida Fish and Wildlife Conservation Commission (FWC). An emergency management plan was later drafted and approved, and a captive-propagation program was initiated (FWC 2003). As of 2008, *C. t. bethunebakeri* is still a candidate to be listed as a federally-endangered subspecies and is the only taxon in Florida that was listed due to public petition.

Captive Colony

The emergency management plan of 2002 (FWC 2003) called for the creation of a captive-propagation program to be housed at the McGuire Center for Lepidoptera and Biodiversity of the Florida Museum of Natural History at the University of Florida in Gainesville, Florida (FLMNH). This colony was initiated utilizing eggs from the BHSP colony in December 2002. The purposes of this colony are to conserve the remaining genetic diversity of the taxon, safeguard the remaining population on BHSP from natural and human factors that may threaten its existence, and produce a significant number of individuals for reintroduction into the wild. As of 2008, 32 captive generations have been produced yielding in excess of 28,000 individuals (J.C. Daniels, unpublished data).

Reintroduction Efforts

With a stable and productive captive colony, efforts began in 2004 to release individuals into suitable habitats in Biscayne National Park (BNP) and Everglades National Park (ENP) as well as Dagney Johnson Key Largo Hammocks Botanical State Park (DJSP). Since May 2004, over 7,500 adults and larvae have been released into these protected parks, areas which are not threatened by urbanization or development. In fact, because of widespread mosquito spraying and habitat destruction outside of the parks, these regions provide a refuge of the only remaining unspoiled beach scrub and hardwood hammock habitat in south Florida. Unfortunately, the extensive damage caused by the 2005 hurricanes has precluded any future releases in ENP. Efforts are currently focused on BNP and DJSP and the extensive, endangered hardwood hammock present on the outlying islands of BNP.

Ecology of *Cyclargus thomasi bethunebakeri*

Life History

Cyclargus thomasi bethunebakeri inhabits tropical hardwood hammocks and their associated margins, beachside scrub and tropical pine rocklands (Minno & Emmel 1993). Of particular interest in this taxon are changes in historical and current use of larval host plants. Historical records list the fabaceous host plants *Caesalpinia bonduc* (L.) Roxb., *C. pulcherrima* L., *Pithecellobium keyense* Britton & Rose, *P. unguis-cati* L., and plants of the family Sapindaceae, *Cardiospermum halicacabum* L. and *C. corindum* L. (Matteson 1930; Klots 1964; Howe 1975; Lenczewski 1980; Brewer 1982; Carroll & Loye 1987; Minno & Emmel 1993; Smith et al.1994; Calhoun et al. 2002; Caroll & Loye 2006). Earlier records (Klotz 1951; Howe 1975) denote *Caesalpinia bonduc* as the larval host plant of this taxon, with references of the last thirty years reporting *Cardiospermum halicacabum* (Lenczewski 1980; Leston et al. 1982; Minno & Emmel 1993; Smith et al. 1994; Calhoun et al. 2002). There are numerous records

from museum specimens and private collectors of larvae in Florida feeding on *Cardiospermum halicacabum* (Minno & Emmel 1993; Smith et al.1994; Calhoun et al. 2002), and possibly *Cardiospermum corindum*, but there is some confusion between the plant taxa and larval feeding (Carroll & Loye 2006). Carroll & Loye (1987; 2006) and Carroll et al. (2003) have studied *Cardiospermum spp.* distribution in Florida and have documented feeding on this genus by other insects. They report that although other authors report *C. t. bethunebakeri* larvae feeding on *Cardiospermum halicacabum* that it is more likely that larvae were feeding upon *Cardiospermum corindum*, a native woody perennial. The species *C. halicacabum* has been considered an agricultural pest, and active eradication plans in Florida have removed many of the plants form the natural landscape. *Cardiospermum corindum* fruits year round and are predominant in the Upper Keys of Florida, making it the likely host plant of *C. t. bethunebakeri* larvae rather than *C. halicacabum*. Carroll & Loye (2006) also report adult characters of the host plant key out to *C. corindum* (Wunderlin 1998). It is likely that authors writing about *C. t. bethunebakeri* larvae have cited past references and perpetuated the taxonomic error in food plant choice in *Cardiospermum* and efforts to locate larvae were focused on this plant and not the previously noted hosts of *Caesalpinia spp*. or *Pithecellobium spp*. In order to feed on *Cardiospermum spp*., *C. t. bethunebakeri* larvae chew a hole into the inflated fruit (hence the common name balloon vine and heart seed vine) and eat the developing seed inside the balloon. *Cyclargus thomasi bethunebakeri* larvae leave their entry hole into the fruit unplugged, allowing for ants to enter the pod to gather sugary secretions from the larvae (Minno & Emmel 1993). The mode of feeding is entirely different for larvae eating the fabaceous hosts *Caesalpinia bonduc* and *Pithecollobium keyense* as larvae feed on developing shoots, young leaves, and flower buds (FWC 2003). Despite extensive surveys, no *C. thomasi bethunebakeri* larvae have

been found on balloonvine since 1992 (J.C. Daniels, personal communication). The current population of *C. t. bethunebakeri* on BHSP only feeds upon *Caesalpinia bonduc* as larvae, even though *P. keyense* is present (FWC 2003). The recently discovered population of butterflies in KWNWR is reported to only feed on *P. keyense* (Canon 2007), and no other host plants are present on these islands. Because of the large taxonomic division between Fabaceae and Sapindaceae and the underlying chemical differences, there has been great interest in the ability of larvae to exploit both host plants.

Cyclargus thomasi bethunebakeri are multiply-brooded on BHSP and individuals can be found throughout the year, although the life cycle does slow in winter months and a slight diapause is suspected (E.V. Saarinen and J.C. Daniels, unpublished data). The generation time is approximately one month. Adult butterflies mate, and females lay eggs on developing shoots, foliage, and flower buds of *Caesalpinia bonduc*. Eggs are laid singly, but may be clustered on developing leaves, shoot tips, and flower buds. Larvae chew out of eggs after several days of development. Larvae develop through four instar stages, the total larval development time lasting 3-4 weeks, with faster development times occurring in warmer and more humid months. Fourth instar larvae pupate in sheltered or inconspicuous areas on *C. bonduc*, often underneath leaf whorls or bracts. Adult butterflies eclose after 5-8 days, again depending on temperature and humidity. Adult butterflies live for 3-7 days in the field, and sex ratios are equal (J.C. Daniels, personal communication). Additional details of life history can be found in FWC (2003).

The *C. t. bethunebakeri* populations on both BHSP and KWNWR are metapopulations, although the two metapopulations do not interact due to over 70 km distance separating the two (E.V. Saarinen and J.C. Daniels, unpublished data). Immature stages are confined to discrete

habitat patch areas of suitable host plant and development is completed in the natal patch. When adult butterflies eclose, they may migrate to nearby, suitable host plant patches in search of mates, food resources, and suitable habitat for egg-laying. This dispersal is usually short-distance as these butterflies are poor fliers (FWC 2003) but adult dispersal does facilitate gene flow between habitat patches. Habitat patches are continuously colonized by gravid females when they are of appropriate quality, and other patches may go extinct when environmental conditions are poor. Thus, there is a dynamic relationship between habitat patches that is driven by environmental factors and the biology of this butterfly. The term *metapopulation* depicts the dynamic nature of both organism movement through the landscape and the changeable landscape itself.

Ant Associations

Historical, anecdotal records of *C. t. bethunebakeri* have mentioned larval associations with the Florida carpenter ant, *Camponotus spp.* Recent studies confirm that larvae associate with *Camponotus floridanus* (Buckley), as well as another member of the genus, *Camponotus planatus* (Roger). Additionally, larvae have been observed tended by *Crematogaster ashmeadi* Emery, *Forelius pruinosus* (Roger), and *Tapinoma melanocephalum* (Fabricius). Field surveys of remaining butterfly habitat and recent reintroduction sites reveal other potential ant associates (*Paratrechina longicornis* (Latreille) and *Paratrechina bourbonica* (Forel)) and a host of potential ant predators (Saarinen & Daniels 2006). Detailed information is also presented about larval ant-associated organs and their mediation of this facultative symbiosis.

Population Genetics

F-statistics

Wright (1951) developed *F*-statistics as a way to describe population genetic structure. These statistics are often used to infer gene flow and evaluate whether a population is in Hardy-

Weinberg equilibrium (HWE). Populations in HWE meet the following assumptions: infinitely large population size, random mating, no mutation, no selection, and no migration. Deviations from HWE may mean that one or more of these conditions are not met and use of *F*-statistics may help determine which variations are due to inbreeding. The F_{ST} statistic has a theoretical minimum of 0.0 and a theoretical maximum of 1.0. Low values indicate little genetic divergence while higher values indicate the fixation of alternative alleles (Hartl & Clark 1997). In 1978, Wright suggested guidelines for interpreting F_{ST} values. F_{ST} values vary considerably among taxonomic groups and studies (review in Frankham et al. 2003), but Wright's guidelines can be utilized for relative comparison of values. He suggested the range 0-0.05 indicates *little* genetic differentiation, 0.05-0.15 indicates *moderate* genetic differentiation, 0.15-0.25 indicates *great* genetic differentiation, and F_{ST} values above 0.25 indicate *very great* genetic differentiation (ital. in Hartl & Clark 1997). Values can also be evaluated whether they are significantly greater than 0. Population differentiation may be underestimated by low F_{ST} values, however, and considerable caution should be used when interpreting results. When population studies utilize microsatellite markers, the statistic R_{ST} is often used in place of or addition to F_{ST}. The former measure specifically considers the stepwise mutation model (SMM) of microsatellite markers and the high rate of mutation in these markers (Slatkin 1995). Additional *F*-statistics include F_{IS} (the correlation between alleles within individuals and the subpopulation) and F_{IT} (correlation between alleles within individuals and the total population).

Founder Effects and Inbreeding

Since Darwin's theories of domesticated species, biologists have been concerned with the effects of inbreeding on organism fitness (Darwin 1876). Early experiments showed the negative impacts of inbreeding due to increased homozygosity on the alleles that affect fitness (Charlesworth & Charlesworth 1999). An alternative hypothesis was also given that inbreeding

depression is due to the expression of deleterious alleles that are unmasked as homozygotes. Wright (1977) showed that heterozygotes have an advantage at loci affecting fitness, and that in randomly mating populations these alleles will be retained as polymorphisms. An excess of homozygotes may occur in non-randomly mating populations, such as those that are inbred. Sibling mating concentrates similar alleles in the same individual, and when these are deleterious recessives, a less fit phenotype is realized and inbreeding depression is observed (Charlesworth & Charlesworth 1987). Additionally, deleterious, recessive alleles may arise in inbred populations via mutation, and these may also negatively impact fitness (Wright 1977). The statistic *f* relates the cumulative amount of inbreeding in a single population. Wright's *F*-statistics and coefficient of inbreeding, *f*, offer standard measures for comparing genetic data for inbred populations.

Inbreeding depression is a major concern in endangered species management (Templeton 2002). As endangered species often exist in small, fragmented populations, instances of inbreeding are often quite high. Gene flow may be severely reduced, and the genetic variation of metapopulations may be exceedingly low. Endangered species may suffer the negative effects of genetic drift, and all members of a population may exhibit decreased fitness measures as a result (Hedrick & Kalinowski 2000). Inbreeding need not always be the culprit of reduced fitness, however. Deleterious alleles may be purged in small, inbred populations, resulting in very well adapted individuals (Byers & Waller 1999). Inbred individuals may be extremely homozygous and less capable of adapting to environmental change, but fitness in the current environment is high. Different species react differently to levels of inbreeding; mammals may exhibit inbreeding depression whereas invertebrates are capable of thriving at similar inbreeding coefficients (Hedrick & Kalinowski 2000; Haikola et al. 2001). It is difficult to make

generalizations about the role of inbreeding in endangered species management plans due to the complex nature and variety of animal and plant genomes. Individual studies are crucial to our understanding of reduced-population mating systems, and the specific factors that affect traits related to fitness need to be examined in individual endangered species (Hedrick & Kalinowski 2000). Current statistical analyses and advances in molecular markers (e.g., microsatellites) have enabled us to detect previously hidden levels of inbreeding depression. Furthermore, inbreeding experiments allow for the direct detection of fitness component reduction in laboratory lines. There is no assumption that inbred lines in the laboratory will behave the same as inbred wild individuals, but a correlation can be inferred (Charlesworth & Charlesworth 1999).

Microsatellites

Microsatellites are highly repetitive, tandem repeats of DNA. They are typically 1-6 base pairs (bp) in length and are found in tandem repeats in (typically) non-coding regions of the genome. Because they are non-coding regions of DNA, microsatellites can retain mutations and exhibit high mutation rates. Differences in the number of microsatellite repeats are useful in determining population structure, heterozygosity, geographic variation, and paternity. They have become the marker of choice in many butterfly population genetic studies owing to the high rate of mutation (Palo et al. 1995; Saccheri et al. 1999; Anthony et al. 2001; Keyghobadi et al. 2002; Harper et al. 2003). Heterozygote deficiencies, geographic variation, and population structure were successfully analyzed with microsatellites in the endangered Karner blue butterfly, *Lycaeides melissa samuelis* (Nabokov), and such studies have set a precedent that genetic studies can be done on endangered species with minimal sampling effect and a great benefit to management plans (Nice et al. 2000; Anthony et al. 2001). Despite its usefulness in population-level studies, the isolation of Lepidopteran microsatellite DNA has been difficult (Zhang 2004)

but other techniques have shown successful amplification of loci using an enriched genomic library (Harper et al. 2000).

Table 1-1. Distribution and records of species of the genus *Cyclargus*.

Species	Species distribution	Reference
ammon	Cuba, Grand Bahamas, Great Abaco, North Andros	Lucas 1857
dominica	Jamaica	Möschler 1886
erembis	Cayman Islands	Nabokov 1948
kathleena	Dominican Republic	Johnson & Matusik 1992
oualiri	Puerto Rico	Brevignon 2002
shuturn	Jamaica	Johnson & Bálint 1995
sorpresus	Dominican Republic	Johnson & Matusik 1992
thomasi	Florida, Bahamas, Turks and Caicos, Hispaniola	Clench 1941
woodruffi	Lesser Antillean islands	Comstock & Huntington 1943

Table 1-2. Subspecies of *Cyclargus thomasi*.

Subspecies	Subspecies distribution	Reference
thomasi (nominate ssp.)	Great Bahama Bank islands	Clench 1941
bethunebakeri	Florida*	Comstock & Huntington 1943
bahamensis	Crooked, Acklin, and Ragged Islands, and Plana Cays, Bahamas	Clench 1942
noeli	Hispaniola (Haiti and Dominican Republic)	Comstock & Huntington 1943
clenchi	Mayaguana, Turks and Caicos, Great and Little Inagua	Miller, Simon, & Harvey 1992

*Smith et al. (1994) note recordings of *C. t. bethunebakeri* from Bimini Islands, Bahamas, but no specimens in current museum holdings have been located to verify this.

CHAPTER 2
DEVELOPMENT AND CHARACTERIZATION OF POLYMORPHIC MICROSATELLITE LOCI

Introduction

The Miami blue butterfly (*Cyclargus thomasi bethunebakeri*) (Lepidoptera: Lycaenidae) is endemic to the state of Florida and, until the 1980s, was relatively widespread and locally common throughout much of the southern portion of the state. Over the last few decades, the butterfly's overall distribution and numerical abundance have been significantly reduced. A variety of factors have been identified as potential causes of decline, including habitat loss, fragmentation and degradation (Calhoun et al. 2002). Reports of verified records rapidly dwindled, and by the early 1990s *C. t. bethunebakeri* was presumed to be extirpated. In 1999, the butterfly was rediscovered as part of a small breeding population in Bahia Honda State Park (BHSP) in the lower Florida Keys. A rapid biological assessment yielded monthly abundance estimates ranging between 35.5 and 141.6 individuals during 2002 and 2003 (J.C. Daniels, unpublished data.). *Cyclargus thomasi bethunebakeri* is currently a state-endangered taxon in Florida and a candidate for federal listing.

As part of a larger conservation effort, a captive-breeding colony was established at the Florida Museum of Natural History's McGuire Center for Lepidoptera and Biodiversity at the University of Florida in Gainesville, Florida. This captive colony was founded with individuals from the Bahia Honda State Park population, and is infused with new genetic material from the same source on a regular basis. Organisms from the captive-breeding colony provide individuals for reintroduction into suitable habitats in south Florida (FWC 2003). The microsatellite markers developed here will be used to analyze both wild and captive-breeding populations as well as newly established colonies resulting from organism reintroductions.

Methods

A microsatellite library was created using the pooled DNA extracts from six adult butterfly thoraxes. These butterflies originated from the captive-breeding colony at the University of Florida, and DNA was extracted with a DNeasy Qiagen Kit shortly after the butterflies expired naturally. DNA was sent to Genetic Identification Services, Inc. (GIS), Chatsworth, CA (http://www.genetic-id-services.com/) for the creation of the microsatellite library. Enrichment was performed for four types of repeats, and GIS used a magnetic-bead capture technology to capture GA, AAT, ATG, and TAGA repeats. A total of 62 different microsatellite-containing clones were sequenced by GIS using universal M-13 primers (forward: 5'-AGGAAA CAGCTATGACCATG -3' and reverse: 5'-ACGACGTTGTAAAACGACGG -3'). DNA sequencing was accomplished using Amersham's DYEnamic™ ET Terminator Cycle Sequencing Kit (Amersham Biosciences P/N US81050), followed by electrophoresis on an Applied BioSystems Model 377 DNA Sequencer. Primers were designed for 33 microsatellite-containing clones using DesignerPCR v. 1.03 (Research Genetics, Inc.). I tested primer pairs against pooled DNA used for microsatellite library development. I then tested twenty-seven candidate loci against seven butterfly individuals, and twelve loci were determined to be polymorphic and suitable for use in genotyping. DNA was extracted from 2 mm^2 wing fragments for genotyping of the 114 Bahia Honda State Park butterflies and stored in 95-100% ethanol at -80°C. Data are presented for all butterflies, representing several breeding populations sampled from September 2005-October 2006. Data is also shown for the September 2005 population; these butterflies were sampled over a five-day period and represent a single breeding population.

PCR conditions for these 12 loci were optimized on a gradient thermocycler (MJ Research PTC-200 Peltier Thermal Cycler). Amplification reactions were performed in a final volume of

10.0 µl containing approximately 2 ng of DNA, 2.5x Eppendorf 5 PRIME MasterMix (1.25 U *Taq* DNA polymerase, 50 mM KCl, 30 mM Tris-HCl, 1.5 mM Mg, 0.1% Igepal®-CA360, and 200 µM of each dNTP), 0.1 mg/ml BSA, and 0.6 µM of each primer. The PCR program consisted of 94°C for 3 min; 34 cycles of 94°C for 40 s, 57°C for 40 s, 72° C for 30 s, and extension at 72° C for 4 min. Primers CthB117 and CthB119 had annealing temperatures of 54°C instead of 57°C. Forward primers were fluorescently labeled at the 5' end with FAM, HEX, or TET (Sigma-Genosys). PCR products were run with GeneScan LIZ-labeled internal size standard (Applied Biosystems, ABI) and analyzed using an ABI 3730 Automated DNA Sequencer and GeneScan 3.1.1 (ABI). Peaks were scored with GeneMapper version 4.0 (ABI) and manually confirmed.

Data were organized in Microsoft Excel and formatted using Convert (Glaubitz 2004) to be used in other software packages. Arlequin version 3.01 (Excoffier et al. 2005) was used to estimate the observed and expected heterozygosities (H_O and H_E) and to test for Hardy-Weinberg equilibrium. FSTAT version 2.9.3.2 (Goudet 2001) was used to test for linkage disequilibrium. Micro-Checker version 2.2.3 (van Oosterhout et al. 2004) was used to test for the presence of null alleles. Sequential Bonferroni corrections for multiple comparisons were performed as per Rice (1988).

Results

Allele numbers varied from four to 14 and ranges of observed and expected heterozygosities were 0.02679-0.79630 and 0.06154-0.69565, respectively (Table 2-1). Only one locus, CthC116, was determined to be in Hardy-Weinberg equilibrium after sequential Bonferroni correction. I performed 66 pairwise comparisons of all loci for linkage disequilibrium using FSTAT, and after a sequential Bonferroni correction, no loci showed evidence of linkage disequilibrium. Null alleles were detected at loci CthB101, CthB106,

CthB117, CthB119, CthC12, CthC116, and CthC124 due to the presence of homozygote excess. There was no evidence of large allele drop-out.

Analysis of a single breeding population of butterflies (N = 24) sampled in September 2005 shows seven loci to be in Hardy-Weinberg equilibrium after Bonferroni correction (Table 2-1). One locus (CthC124) was monomorphic in this population. No loci were in linkage disequilibrium. Null alleles were detected at loci CthB117, CthB119, and CthC12 due to the presence of homozygote excess.

Discussion

The butterflies genotyped for this study were from a very small population, which likely accounts for a significant deviation from Hardy-Weinberg equilibrium at several loci. Homozygote excess was likely the result of generations of inbreeding. Population demographics are currently under investigation and further studies will employ the use of the microsatellite markers described here.

Table 2-1. Characteristics of the twelve polymorphic microsatellite loci developed for *C. t. bethunebakeri*. Primer sequence, *F*=FAM, *H*=HEX, *T*=TET; T_a, optimized annealing temperature; Size range, observed size range of alleles in bp; *N*, number of *C. t. bethunebakeri* successfully genotyped (out of 114); N_A , total number of alleles; H_O and H_E, observed and expected heterozygosities, respectively; *P* HWE (total/ BHSP-9-2005), *P* value for Hardy-Weinberg equilibrium across the whole sample set of 114 individuals (above diagonal) and for the single breeding population (*n*=24) sampled in September 2005 from Bahia Honda State Park (below diagonal), those in italics are not in equilibrium.

Locus Name	GenBank Accession no.	Repeat motif	Primer sequence (5'-3')	T_a	Size range (bp)	*N*	N_A	H_O	H_E	*P* HWE (total / BHSP-9-2005)
CthB11	EU429568	$(ATT)_{12}$	F:*F*-GTGACGAAAAGATAGCAACAG R:TTCCATCAGGTGACTCCTC	57	150-216	108	7	0.79630	0.69565	*0.00040 /* 0.03049
CthB101	EU429569	$(AAT)_{10}$	F:*H*-TCACTGATGCTCGTCTCTCAC R:CCGAACCGTTGGTAGGTC	57	138-201	107	6	0.51402	0.66237	*0.00000 /* 0.28371
CthB103	EU429570	$(AAT)_{10}$	F:*T*-CTCCGAGATCACTCGAACT R:CGGAAATGAACCATGTTG	57	150-233	113	6	0.49558	0.57278	*0.00000 /* 0.16213
CthB106	EU429571	$(ATT)_8$	F:*H*-CTGAGCCTAGTTTAGACTCCAG R:GGGATTTGAGAATGGTTTAGA	57	163-208	108	7	0.31481	0.41796	*0.00000 / 0.00000*
CthB115	EU429572	$(ATT)_{11}$	F:*F*-TGGTGTGGTGTAAAGTATGTCA R:TCATGGTTCCTATCATCAGC	57	292-441	111	7	0.45946	0.59989	*0.00000 /* 0.09258
CthB117	EU429573	$(ATT)_{21}$	F:*H*-CAGGGTAGGTAAATGCGTGTC R:GCTTTCCATCAGGTGACTCC	54	152-189	114	9	0.20175	0.34222	*0.00000 / 0.00000*
CthB119	EU429574	$(AAT)_{11}$	F:*F*-AGGGTAGGTAAATGCGTGTC R:CTTTCCATCAGGTGACTCCT	54	135-186	110	10	0.18182	0.31802	*0.00000 / 0.00034*
CthC12	EU429575	$(GAT)_6$	F:*T*-GATCCGATTGCCTCTATCG R:TTACGCCTCATTCTTAACATCC	57	171-228	113	14	0.31858	0.65971	*0.00000 / 0.00076*
CthC116	EU429576	$(CAT)_{17}$	F:*T*-TGCGAAACTACAGTCACCTC R:AAGACCATCCAAGAAACCAC	57	242-251	112	4	0.25000	0.31126	0.10107 / 0.57871
CthC124	EU429577	$(CAT)_{16}$	F:*H*-GAACCACTGACCTCGTAACATG R:TGCAGGAAGGATAAACAAACAC	57	230-248	112	5	0.02679	0.06154	*0.00000 / Monomorphic*
CthC127	EU429578	$(GAT)_{11}$	F: *F*-AAATCGTGATTAGCTGTCGA R:TAAGGAACTCGGCATCATG	57	191-206	108	5	0.49074	0.60168	*0.00261 /* 0.02057
CthD7	EU429579	$(TATC)_{24}$	F:*H*-TGGTAGAGCCGATTTAGGTG R:CAACCAGCTCAACTAACCAAC	57	221-313	110	13	0.59091	0.69124	*0.00000 /* 0.17686

CHAPTER 3
UTILIZING MUSEUM SPECIMENS TO ASSESS HISTORICAL DISTRIBUTION AND GENETIC DIVERSITY

Introduction

The results of conservation programs for imperiled species are substantially improved when appropriate ecological and genetic information are incorporated into research and management plans (IUCN 2008). However, even for historically widespread and abundant species, sufficient data may not be available prior to the decrease in the geographic range or population size of the focal taxa. Such population declines are frequently concomitant with losses of genetic variation that often occur first as responses to these declines and then as negative feedback that result in further decreases in population size (Frankham 2003). Consequently, conservation efforts may be severely hindered by both insufficient information on the current status of imperiled species and imprecise or entirely absent data regarding ecological and genetic aspects of extinct populations.

A historical approach to reconstructing the ecology of imperiled taxa can offer invaluable knowledge regarding the geographic and genetic composition of these lost populations (Leonard 2008). Museum collections can be an important resource for such approaches (Harper et al. 2006; Winston 2007). Insect collections are generally kept at low relative humidities and constant temperatures, conditions which improve DNA preservation (Mandrioli et al. 2006; Leonard 2008). Some researchers have reported success in genotyping museum specimens of insects (Goldstein & DeSalle 2003; Harper et al. 2003, 2006) whereas others have questioned the utility of such methods (Watts et al. 2007). However, recent advances in molecular genetic methods greatly improve the success of such endeavors (e.g., Harper et al. 2006). Additionally, the study of museum specimens allows researchers to plot historical occurrences of specimens, often with sufficient coverage to infer geographic range, and to identify potential changes in

populations over time. Indeed, combining knowledge of an imperiled taxon's historical distribution from specimen label data with genetic analysis of the corresponding specimen is a powerful new approach for understanding the spatio-genetic structure of historical populations and may therefore have substantial contributions for directing future research and conservation efforts.

The Miami blue butterfly (*Cyclargus thomasi bethunebakeri*) was historically common in the Florida Keys and southern Florida and anecdotal records show that its range extended northward along both coasts to Hillsborough and Volusia Counties (Forbes 1941; Kimball 1965; Calhoun et al. 2002; Carroll & Loye 2006). Amateur and professional naturalists regularly encountered this butterfly in localized patches in south Florida, even referring to it as a nuisance when looking for rarer butterfly taxa (C. Covell, I. Finklestein, and R. Gilmore, personal communications). However, *C. t. bethunebakeri* populations began to disappear in the 1970s and by the 1980s it became increasingly difficult to find the butterfly even in apparently high-quality habitat in southern Florida. By 1990 the butterfly had become considerably rarer and, with no verified records from March 1992 to October 1999, was thought to be extinct (Calhoun et al. 2002). A small colony of butterflies was rediscovered in November 1999 within the boundaries of Bahia Honda State Park (BHSP) on Bahia Honda Key in the lower Florida Keys (Ruffin & Glassberg 2000). In December 2007, an additional population was discovered on several uninhabited islands within the Key West National Wildlife Refuge (KWNWR) (Cannon 2007). Despite formerly high population abundance and extensive geographic range throughout coastal southern Florida, wild populations of *C. t. bethunebakeri* are currently limited to a few small insular areas in the extreme southern portion of the state.

A variety of anthropogenic and natural factors likely led to the decline of *C. t. bethunebakeri*, with habitat loss, degradation and fragmentation driven by development and human population growth undoubtedly playing key roles. The human population of the state has increased from fewer than a million people in 1920 to over 18 million in 2007 (U.S. Bureau of Census, www.census.gov/population/cencounts/fl190090.txt). Dade and Monroe Counties, two areas in which the butterfly was historically abundant, have exemplified this rapid growth (Table 3-1). Land use and land cover change have been correspondingly dramatic in this region, with agricultural and urban land currently predominating (Solecki 2001). Solecki (2001) divided the changes in south Florida land use into three eras: the frontier era (1870-1930), development era (1931-1970), and globalization era (1971-present) based on characteristics of political and economic changes occurring in the region. The greatest land cover changes in Florida took place in the development era from 1953-1973 with the conversion of 5,800 km^2 of natural areas to agricultural and urban use areas (289.97 km^2/year) (Solecki & Walker 2001). Habitat loss and fragmentation cannot be singled out as the sole causes of decline of *C. t. bethunebakeri* as there are a multitude of unknown factors in operation. Additionally, natural forces have impacted remaining areas of habitat suitable for *C. t. bethunebakeri* as evidenced by several major hurricanes in the 1920s and 1990s and extreme drought in the 1960s (Solecki 2001). The synergistic effects between habitat loss and the unquantifiable losses from predation, parasitism, competition from invasive species, and other unidentified factors should not be discounted or overlooked (Calhoun et al. 2002).

The goal of this study was to examine how changes in the population size and geographic distribution of *C. t. bethunebakeri* have affected the genetic diversity of this historically widespread taxon that has experienced dramatic decreases in range and population size.

Specifically, I used ancient DNA techniques to assess changes in genetic diversity in a single population over time and compared historical genetic diversity with that found in the two remaining populations. Similar approaches have proven useful for other conservation projects (Ross et al. 2006; Leonard 2008). My study was motivated by the need to determine historical levels of genetic diversity over time in order to better understand and preserve the current genetic diversity present in extant populations. This study has further addressed whether the size of the butterflies have changed over time, season, and location, factors which may speak to the evolutionary potential and plasticity of the taxon.

Methods

Collection Database

I directly transcribed or requested label data from *C. t. bethunebakeri* specimens held by five museums with preeminent Lepidoptera collections: the McGuire Center for Lepidoptera and Biodiversity at the Florida Museum of Natural History, Gainesville, Florida (FLMNH) (*N* = 361); the American Museum of Natural History, New York City, New York (AMNH) (*N* = 215); the Carnegie Museum of Natural History, Pittsburgh, Pennsylvania (CMNH) (*N* = 179); the Smithsonian National Museum of Natural History, Washington, D.C. (SNMNH) (*N* = 108); and the Field Museum, Chicago, Illinois (CNHM) (*N* = 14). All specimens were verified in consultation with taxonomic experts as being *C. t. bethunebakeri.* Taken together, label data were used to assess and develop the distributional range of the butterfly over time.

I used label information from the collection database to create a GIS database for *C. t. bethunebakeri*. Label data were georeferenced using Google Earth 4.3 (http://earth.google.com/) and then converted into a geodatabase using the "convert kml to shp" tool from ArcGIS 9.2 (Environmental Systems Research Institute – ESRI, Redlands, California). Distribution maps were created in ArcGIS 9.2. Not all label data from museum collections were usable for

distribution-level analyses. Some labels were unreadable and others did not give a year or specific location record. Museum staff limitations prevented other data from being recorded beyond the county level. The total number of usable *C. t. bethunebakeri* collection data records was $N = 689$. Collection records were divided into three eras: the frontier era (1870-1930), development era (1930-1970), and globalization era (1970-present) as per Solecki (2001), and plotted on base maps to assess differences in specimen records over time.

Data from FLMNH specimens were also used to examine relationships between body size (wing chord length), sex, and time and location of collection. Temporal data from collection records were analyzed by decade and by south Florida season (wet vs. dry). Wet seasons were delineated as months having more than 3.2 inches of rainfall and 76°-90°F average temperatures using data from National Oceanic and Atmospheric Administration (NOAA) data for Key West, Florida (http://www.srh.noaa.gov/key/HTML/climate/eywclimate.html) (Figure 3-1). The months May-October were scored as wet months and dry months were January-March and November-December. Location data were organized both on a county and a regional level. At the regional level, locations were divided into seven groups: the lower Keys, the upper Keys, Everglades National Park, the Miami area, the Ft. Lauderdale area, the Naples area, and Sanibel Island. Data were also analyzed by grouping samples into "eastern mainland" and "Keys" populations, the former group encompassing samples from Everglades National Park, the Miami area, and the Ft. Lauderdale area and the latter group being comprised of samples from all of the Florida Keys.

Wing chord measurements (forewing length) from the historical FLMNH samples were also compared with all current BHSP samples (2002-2003 and 2005-2006) to assess changes in

wing length by sex, season, and year. ANOVA tests were run to assess relationships between wing chord length, sex, season, year collected, and location of collection in Microsoft Excel.

Genetic Sampling

I selected museum samples (FLMNH only) for genetic analysis based on two criteria. First, wide geographic and time ranges were required to maximize detection of allelic diversity over space and time. A broad geographic coverage is represented by samples FLMNH_004-FLMNH_008 (Table 3-2). These five samples formed the "mixed" group and were from multiple locations collected from 1966-1988. Second, numerous specimens taken over a series of days in the same location were necessary to identify individual population signatures. For these signatures, I selected three groups of samples taken from Key Largo, Florida, which has been noted as a former "hotspot" for this taxon (J. Calhoun, personal communication.). I found no historical specimens recorded from Bahia Honda State Park or Key West National Wildlife Refuge, the two sites with extant *C. t. bethunebakeri* populations, therefore, the extensive collecting on Key Largo provided the best possible sampling to allow me to examine the change in a single population over time. The 1940s location represented eight butterflies collected in Key Largo in May in the 1940s. Label data for this group was indicated as "194_" by the collector, so the exact year is unknown. The 1963 population was represented by 14 organisms collected over two days in April 1963 on Key Largo. The last ten samples were collected from a population of *C. t. bethunebakeri* on North Key Largo on a single day in March 1979. These three sample groups presumably represented genetic diversity of a temporally continuous Key Largo population and allowed me to examine temporal changes in genetic structure at this site.

DNA Extractions and Microsatellite Analyses

DNA was extracted from museum specimens in a dedicated ancient DNA laboratory at the University of Florida's Florida Museum of Natural History (FLMNH) and followed special

protocols to prevent contamination with contemporary DNA. Negative controls were run with every extraction to ensure that current material did not contaminate museum samples. A maximum of fifteen extractions were carried out at a time to minimize contamination between samples. All samples were well labeled to keep data matched with museum samples. I carefully removed 1-2 adult butterfly legs and labial palps from FLMNH museum specimens. Extraction methods followed the Qiagen DNeasy Blood and Tissue Kit protocol with modifications for historical material from Iudica et al. (2001). Butterfly legs and palps were washed three times with a 250µl 1X PBS for 10 minutes at 55°C for each wash. After washing, I added 180µl Buffer ATL and ground up the sample using a sterile, disposable pestle. I then added 20µl Proteinase K, mixed by vortexing, and incubated at 55°C in a rotating rack for 24-48 hours. The remainder of the protocol followed the Qiagen Kit, with the addition of carrier nucleotides (yeast tRNA) to force precipitation as outlined in Kishore et al. (2006). DNA was eluted one time with 30µl warm Buffer AE. I conducted PCR amplifications on ancient DNA templates in another dedicated laboratory using UV-sterilized equipment and laboratory bench top to prevent contamination from current materials. PCR conditions for twelve polymorphic microsatellite loci followed those of Saarinen et al. (2009), with the modification that 5µL of template DNA were used per 25 µL PCR reaction and there was no multiplexing or multi-pooling of samples.

Data for current population genetic diversity were gathered from two different generations from the BHSP population (September 2005 and June 2006) as well as from the Marquesas Islands in KWNWR (February 2008). DNA extraction and PCR amplifications on contemporary samples followed Saarinen et al. (2009).

Only one locus (CthB119) was successfully amplified from historical DNA in the majority of samples and was therefore the only locus used for analysis. Microsatellite data from locus

CthB119 from contemporary populations (BHSP and KWNWR) were checked for null alleles using Micro-Checker version 2.2.3 (van Oosterhout et al. 2004) and significance was assessed after a Bonferroni correction (Rice 1988). All populations were tested for Hardy-Weinberg equilibrium (HWE) in Arlequin version 3.01 (Excoffier et al. 2005) and a sequential Bonferroni correction was performed across multiple comparisons. As data from a single locus are presented, no linkage disequilibrium tests were performed. Data from a previous study of *C. t. bethunebakeri* on BHSP showed that no linkage exists between any of the twelve microsatellite markers described for this taxon (Saarinen et al. 2009).

Analyses of observed (H_O) and expected (H_E) heterozygosity were calculated using Arlequin 3.01 (Excoffier et al. 2005) and an analysis of the H_O/H_E ratio was graphed to assess changes over time. The number of alleles (N_A) and number of effective alleles (n_e) were calculated using GenAlEx 6.1 (Peakall & Smouse 2006). The calculation of effective population size (N_E) requires either knowledge of consecutive generations of allelic data or knowledge of sex ratios, family size variance, and population size fluctuations (Frankham et al. 2003; Wang & Whitlock 2003) or data from multiple loci for linkage disequilibrium estimates (England et al. 2006; Tallmon et al. 2008). Given these limitations, I have instead opted to compare the effective number of alleles (n_e) of different populations over time, calculated as

$$1/ (\Sigma p_i^2) \qquad (3\text{-}1)$$

where p_i is the frequency of the i^{th} allele for the population (Peakall & Smouse 2006). Lastly, I used GenAlEx 6.1 to estimate genetic divergence between populations using F_{ST} (Weir & Cockerham 1984) and R_{ST} (Slatkin 1995) and significance levels were calculated using 999 permutations.

Results

Geographic Distribution of Museum Specimens

I examined 877 *C. t. bethunebakeri* specimens from museum collections, of which 689 had sufficient label data for inclusion in my analyses. A large, primarily coastal, geographic distribution of this butterfly is evident (Figure 3-2, 3-3, 3-4). During the early part of the century, most specimens were collected from the Miami area, with a few inland and northern county records, and no records from the Florida Keys. Specimens from mainland Monroe County as well as the Florida Keys began to appear in the 1930s, but most records during the development era were still from the Miami area and Dade County. During the globalization era (1970-present) the Florida Keys became the major collection location, with very few records from Dade County. Specimen counts for eleven counties over time (1900-1990) showed that most specimens were collected in Dade and Monroe Counties. (Figure 3-5). Records from Dade County (N = 212) (which included the Miami area) were most numerous in the 1930s and 1940s, and Monroe County (N = 387) records (including all of the Florida Keys) were most numerous in the 1970s (Figure 3-6).

Relationships Between Size, Sex, Year, and Collection Location of Museum Specimens

There was a significant difference in wing chord length between male (N = 206) and female (N = 132) *C. t. bethunebakeri* ($F_{1, 336}$ = 6.344, P = 0.012) from the FLMNH collection. Males were smaller than females, with respective wing chord lengths of 11.221mm ± 0.075 (mean ± SE) and 11.515mm ± 0.088 (mean ± SE). There was no significant difference in wing chord length between wet (N = 146) and dry (N = 192) seasons ($F_{1, 336}$ = 0.932, P = 0.335), nor was there a significant effect of the decade of collection on wing chord length (1920s N = 15, 1930s N =13, 1940s N = 33, 1950s N = 8, 1960s N = 58, 1970s N = 141, 1980s N = 55; F6, 316= 0.913, P = 0.486). There were no significant differences in wing chord length between the seven

different location regions (Lower Keys $N = 58$, Upper Keys $N = 196$, Everglades National Park $N = 15$, Miami area $N = 56$, Ft. Lauderdale area $N = 7$, Naples area $N = 4$, Sanibel Island $N = 2$) ($F_{6, 331} = 1.27$, $P = 0.270$). There were also no significant differences between eastern mainland Florida populations (Everglades National Park, Miami, Ft. Lauderdale $N = 78$) and specimens collected in the Florida Keys ($N = 241$) ($F_{1, 318} = 0.022$, $P = 0.882$). Lastly, there were no seasonal size differences between the mainland Florida populations and those in the Keys either during the dry season ($F_{1, 176} = 0.659$, $P = 0.418$) or between groups in the wet season ($F_{1, 141} = 0.405$, $P = 0.526$).

Comparison of Body Size in Historical and Current Populations

The historical *C. t. bethunebakeri* specimens from FLMNH were compared with butterflies from BHSP (2002-2006) to determine whether there has been a change in wing chord length between historical populations and the extant BHSP population. Initial assessment of the BHSP population itself showed that females were significantly larger than males (for individuals measured in the wild, not reared in the laboratory) ($F_{1, 124} = 21.124$, $P < 0.001$) (Table 3-3). Individuals sampled from BHSP in the wet season were also significantly larger than dry season BHSP adults ($F_{1, 235} = 19.923$, $P < 0.001$). When the BHSP data were compared to FLMNH data, there were several significant differences in wing chord lengths. BHSP individuals (11.585 ± 0.054) were significantly larger than historical samples (11.297 ± 0.058) ($F_{1, 599} = 12.900$, $P < 0.001$). Females from BHSP were also significantly larger than historical samples of females ($F_{1, 233} = 12.756$, $P < 0.001$) and BHSP adults measured in wet seasons were larger than those sampled in wet seasons in museum collections ($F_{1, 347} = 17.681$, $P < 0.001$) (Table 3-3).

Historical Genetic Diversity

I attempted PCR-based amplifications on DNA extracted from museum samples at eight of the 12 microsatellite loci used to genotype modern specimens. However, only one locus

(CthB119) successfully amplified in the majority of historical DNA samples and was therefore the only locus used for analysis. Loci CthB11 and CthB117 amplified for a few samples, whereas CthB101, CthB103, CthB106, CthC124, and CthD7 failed to amplify for any samples. I lacked sufficient quantities of historical DNA templates to test the remaining four microsatellite loci.

Null alleles were detected in both contemporary populations (BHSP and KWNWR) at locus CthB119. Both contemporary populations showed homozygote excess but no signs of scoring error or large allele drop-out. Analyses of allelic data showed that Hardy-Weinberg equilibrium (HWE) values at locus CthB119 were different between historical and contemporary populations. All historical populations were in HWE but no contemporary populations were in equilibrium (Table 3-4). Data from the twelve microsatellite loci developed for this taxon (Saarinen et al. 2009) were considered for the contemporary populations, and these results show that seven of 12 loci were in HWE in the September BHSP 2005 population, six of 12 in HWE in the June BHSP 2006 population, and ten of 12 in HWE in the February 2008 KWNWR population after Bonferroni correction. I also plotted the ratio of H_O/H_E for populations from each time period (Figure 3-7). This ratio in locus CthB119 is consistent in the historical populations, but is lower in 2005. The 2006 and 2008 populations show ratios similar to historical levels. The additional 11 microsatellite loci analyzed in the contemporary populations show a range of H_O/H_E ratios between loci and years. The average ratios (across 12 loci) in each of the contemporary populations are similar to the ratios generated from the single locus CthB119 in the historical populations (Figure 3-7).

Even with very small sample sizes, the historical populations had generally higher effective number of alleles (n_e) and observed levels of heterozygosity (H_O) than the extant BHSP

population (Table 3-4). Additionally, the effective number of alleles and observed heterozygosity values remained relatively stable or even increased across the three sampling periods in the historical Key Largo population (1940s, 1963, and 1979). The BHSP population showed reductions in both the effective number of alleles and observed levels of heterozygosity when compared to historical levels. The KWNWR population values were between historical levels and BHSP values (Table 3-4).

Four newly detected alleles (135, 165, 171, and 180) were found in very low frequencies in the BHSP populations. Historical populations showed a more diverse distribution of allelic frequency than the current BHSP population (Figure 3-8). The BHSP populations had one main allele (162) and low frequencies of other alleles. Historical populations had a greater distribution of allelic frequencies across the alleles sampled thus far at locus CthB119 (Figure 3-9).

Allele 132 was only sampled from the KWNWR 2008 population (Table 3-5). Alleles 135, 153, 165, and 171 were only found in one of the BHSP populations, and all were at very low frequency. Only one allele (147) found in historical samples was not found in either the BHSP or KWNWR populations. This allele was present in three of the four historical populations with relatively high frequency (0.082-0.167) but was absent from both current populations (Table 3-5). Allele 177 was present at a higher frequency in two historical samples (0.125-0.250) as well as in the KWNWR population (0.229), and was not detected in either BHSP sample. Allele 156 was also present in the Key Largo 1963 population (0.063) and KWNWR population (0.042), but was again rare in BHSP (0.028) and only found in the 2006 samples. Allele 180, however, was found in all contemporary, but no historical population samples and allele 186 was found in all historical samples and KWNWR, but neither BHSP sample. Overall the BHSP and KWNWR populations shared four of 11 alleles with frequencies

0.020 and above (156, 159, 162, and 180). The KWNWR and historical populations shared five of eleven alleles when measured under the same criterion (156, 159, 162, 177, and 186).

Genetic divergence between historical and contemporary populations is evident from both R_{ST} and F_{ST} measures (Table 3-6). The BHSP samples are significantly different from all historical populations as well as the extant KWNWR population ($P < 0.001$). The KWNWR does not show significant differences from any of the historical populations.

Discussion

This study showed that *C. t. bethunebakeri* specimens were common in museum collections from the early 1900s to the 1980s. The distribution of these butterflies over south Florida over time was further verified through examination of museum records and this wide distribution showed that butterflies were able to exist in a variety of locations in southern Florida and that they were found in high numbers in local patches. Collection records for Dade Co. (location of Miami, the butterfly's namesake city) were highest in the 1930s and 1940s, prior to massive land use change and urbanization (Solecki 2001). Monroe Co. records increased in later decades, peaking in the 1970s. Decreases in natural land areas could not be correlated with changes in the number of butterflies collected (or butterfly population abundance) as there were several confounding factors involved. Growing human populations not only lead to a decrease in natural land areas but an increase in pesticide use for mosquito control and agriculture. Land use changes may also indirectly impact native fauna by increasing the ranges of invasive plant and animal species (Chapin et al. 2000; Carroll & Loye 2006), and increased competition may further depress butterfly population numbers. Land management practices including fire suppression and exotic species removal further affect remaining habitat and none of these factors should be underestimated nor pinpointed as the sole cause of decline of native taxa.

It was not possible to quantify issues of collector bias when assessing the relative abundance of historical specimens. Because of the wild nature of the Florida Keys, collecting restrictions, and the inaccessibility of certain islands, it was understandable why certain regions were visited more heavily than others by collectors. Additionally, certain areas may have been targeted for collecting in order to observe other localized or rare species. Key Largo was one such location, and it was uncertain whether this island represented a "central hotspot" of the historical *C. t. bethunebakeri*, a spot simply heavily visited by lepidopterists, or a combination of the two. The island of Key Largo may have represented one of several population centers and one that was easily accessible to collectors due to the road network. Other regions, especially those within protected lands like Everglades and Biscayne National Parks, may have held additional populations of butterflies that were not as heavily sampled by collectors.

Analyses of wing chord data confirmed that females were larger than males in both historical and contemporary populations. Additional differences between historical and recent specimens showed that females from BHSP were larger than historical female specimens and that individuals from BHSP sampled in the wet season were larger than historical specimens sampled during wet seasons. Overall analyses proved that BHSP individuals were larger than historical butterflies. These results may reflect that larger adults were positively selected for over time. Larger adult butterflies may be more capable of dispersing and finding food and mates. Larger adult size has also been correlated with increased fecundity and fitness, although this is only one measurement of potential fitness (Calvo & Molina 2005). Smaller adult size in butterflies is usually associated with limited food resources during larval development or abrupt termination of availability of food in the last larval instar, both conditions resulting in early pupation and a smaller adult size (T.C. Emmel, personal communication).

PCR reactions failed to amplify 7 of 8 tested microsatellite loci in all individuals and additional loci could not be tested due to a paucity of historical DNA templates. There were several possible reasons for the failure of PCR amplification on historical templates. Historical DNA template may have been too degraded or the region to be amplified may have been too long to be amplified in sheared DNA. These issues may be addressed by re-designing primers to amplify shorter regions of interest, as has been done for larger bar-coding endeavors (Hajibabaei et al. 2006). The length of microsatellite repeats may prevent this option at some microsatellite loci, but it may be possible at others. The utilization of single nucleotide polymorphisms (SNPs) to analyze historical specimens may represent a better approach for the re-creation of historical population signatures (Goldstein & DeSalle 2003; Morin & McCarthy 2007; Svensson et al. 2007). A genomic approach to conservation genetics is also becoming increasingly possible with the advent of affordable large-scale sequencing technology (Leonard 2008).

The genetic differences between the contemporary and historical populations were significant, but these should be interpreted carefully as they were from a single microsatellite locus. Although other studies reconstructing historical populations have utilized three or four microsatellite markers (Hutchinson et al. 2003; Harper et al. 2006) the single locus used in this study did contain eleven alleles including several rare alleles. These alleles are useful in detecting genetic drift and as such, provide useful data (Frankel & Soulé 1981). Measurements of HWE showed that historical populations remained in HWE over time and that the contemporary populations were in HWE at some loci.

The temporal sampling employed by this study supports the conclusions that historical populations contained more genetic diversity than the extant BHSP population. This conclusion is supported by several different genetic measurements (N_A, n_e, H_O, H_E), all of which suggest that

historical populations were more diverse than either contemporary population. The historical populations showed higher observed heterozygosity than either of the contemporary (BHSP and KWNWR) populations. The number of effective alleles of historical populations was also higher, even with the limited sampling. Current overall allelic frequencies were reduced, with the BHSP population showing higher frequencies of fewer alleles. Because of the intensive sampling of the BHSP population, there was a greater presence of low frequency alleles. There were 12 total alleles at CthB119, and three of these were only found in the BHSP population with 0.014 frequencies. These may represent novel mutations as they were not found in the historical populations or in the KWNWR population, although it is equally possible that they were simply not sampled in those groups. Only one allele was found in historical populations but was absent from both BHSP and KWNWR populations. This allele (147) was historically present at a relatively high frequency (0.083-0.167). These data show that although allelic frequencies have changed over time, only one allele has not been found and is presumed to be lost from contemporary populations. Other studies comparing historically-common and currently-endangered populations have shown a greater loss of alleles over time (Harper et al. 2006).

The KWNWR population, found on the Marquesas Islands, had higher genetic diversity measures than the BHSP population, and although little is known of this new population, it does lend encouragement for future conservation efforts. Both historical and contemporary populations show evidence of a metapopulation structure with interacting sub-colonies (E.V. Saarinen and J.C. Daniels, unpublished data). The metapopulation structure on KWNWR is more extensive than the metapopulation structure present on BHSP, potentially providing better methods for retaining genetic diversity. Further review of population structure and the

importance of metapopulation dynamics in this taxon are forthcoming and will be incorporated into management strategies.

Overall patterns of genetic diversity in the BHSP population (mean overall observed heterozygosity of 39.5%) are similar or slightly lower when compared with other non-migratory butterfly species' studies utilizing microsatellite markers. Butterfly species that are not considered endangered show a range of 40.97-48.37% mean overall observed heterozygosity (Keyghobadi et al. 2002; Fauvelot et al. 2006; Sarhan 2006). This study is not the first to show high levels of genetic variation in an endangered taxon. The Mauritius kestrel (Groombridge et al. 2000) and the greater one-horned rhinoceros (Dinerstein & McCracken 1990) have both recovered from extreme bottlenecks and show high heterozygosity without the addition of new genetic material. Studies of an isolated population of the brown bear *Ursus arctos* also demonstrated high heterozygosity despite a severe, well-documented bottleneck (Hartl & Hell 1994). All of these studies highlight the fact that bottleneck events resulting in low population numbers and the reduction of genetic diversity do not necessarily doom endangered taxa to extinction and that these taxa should not be "written off" as lost causes or denied funding on such grounds. This research speaks to the importance of studying historical populations in making assumptions of current populations (Leonard 2008; Shepherd & Lambert 2008), but grand assumptions cannot and should not be made from the frequency of alleles at a single locus. Furthermore, data characterizing historical populations should be calculated from numerous museum specimens to better estimate levels of historical diversity.

This study showed that museum collections represent a wealth of information that may prove invaluable for understanding the distribution of rare taxa and may be useful for conservation planning. Butterflies are particularly well-represented in museum collections and

significant taxonomic and life history information is available for Lepidoptera, making it ideal for studies such as this one. Similar historical approaches may be possible for a wide range of taxa that have small extant populations complemented by large museum holdings from extinct populations. The extant *C. t. bethunebakeri* populations are geographically restricted to a few small islands in the Florida Keys and do not include the full genetic diversity formerly present when populations were more widespread and abundant. I have demonstrated here that the historical distribution and genetic variation of extinct populations can be reconstructed by careful and thorough examination of museum specimens.

The habitat changes that have occurred in south Florida are neither unique nor reversible, but that does not mean that species conservation efforts cannot be successful in this region. Between the two contemporary populations, *C. t. bethunebakeri* has retained a significant amount of genetic diversity from its historical values. Through active and timely conservation efforts, this taxon holds promise to be restored to its once common status. This highlights the need for conservation of the current island populations as they represent the last members of the taxon and the remainder of historical diversity. Conservation efforts for this taxon should not be abandoned on the grounds of reduced molecular diversity or adaptive ability, as the genetic variation shown here is evidence of evolutionary potential. Other taxa with reduced population sizes should be genetically assessed and compared to historical populations through the analysis of museum specimens whenever possible. With proper sampling and methodologies, such projects can assess historical levels of gene flow and estimate appropriate levels for contemporary populations. The utilization of museum label data and historical genetic analyses can also be incorporated into conservation studies in recommending new areas for reintroduction (Ross et al. 2006). These efforts may help restore endangered species to common status by

giving historical perspective, offering guidelines for reintroduction efforts and genetic management, and providing a context for interpreting present-day genetic diversity.

Table 3-1. Human population estimates in south Florida counties. Source: U.S. Census Bureau 2008.

Year	Monroe	Dade	Florida total
1900	18,006	4,955	528,542
1910	21,563	11,933	752,619
1920	19,550	42,753	968,470
1930	13,624	142,955	1,468,211
1940	14,078	267,739	1,897,414
1950	29,957	495,084	2,771,305
1960	47,921	935,047	4,951,560
1970	52,586	1,267,792	6,789,443
1980	63,188	1,625,781	9,746,324
1990	78,024	1,937,094	12,937,926
2000	79,589	2,253,362	15,982,378
2007	73,223	2,387,170	18,251,243

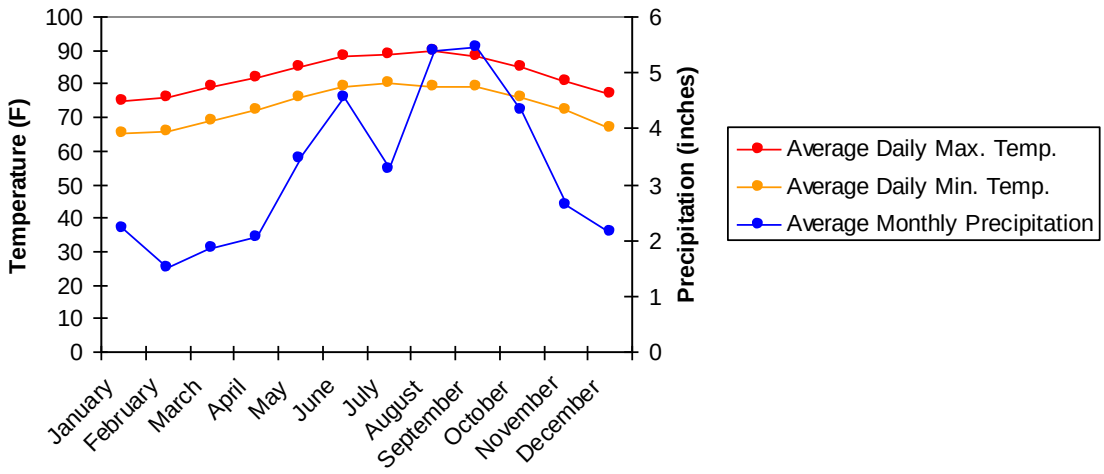


Figure 3-1. Weather data averages for Key West, Florida.

Table 3-2. Museum specimens of *C. t. bethunebakeri* sampled for genetic diversity. Samples FLMNH_009-FLMNH_016 had data missing from the museum label. The data listed on the museum specimen labels are given in the table. Population group relates to the assignment of specimens for statistical analyses.

Sample ID	Location	Date	Population Group
FLMNH_004	Middle Cape Sable, Everglades Nat'l. Park	April 7, 1966	Mixed
FLMNH_005	Biscayne Nat'l. Monument	May 10, 1973	Mixed
FLMNH_006	Plantation Key	March 1, 1981	Mixed
FLMNH_007	Little Pine Key	April 26, 1988	Mixed
FLMNH_008	Big Pine Key	May 29, 1979	Mixed
FLMNH_009	Key Largo	May 17 194_	1940s
FLMNH_010	Key Largo	May 17 194_	1940s
FLMNH_011	Key Largo	May 17 194_	1940s
FLMNH_012	Key Largo	May 18 194_	1940s
FLMNH_013	Key Largo	May 18 194_	1940s
FLMNH_014	Key Largo	May 18 194_	1940s
FLMNH_015	Key Largo	May 18 194_	1940s
FLMNH_016	Key Largo	May 31 194_	1940s
FLMNH_017	Key Largo Key	April 2, 1963	1963
FLMNH_018	Key Largo Key	April 2, 1963	1963
FLMNH_019	Key Largo Key	April 1, 1963	1963
FLMNH_020	Key Largo Key	April 1, 1963	1963
FLMNH_021	Key Largo Key	April 1, 1963	1963
FLMNH_022	Key Largo Key	April 1, 1963	1963
FLMNH_023	Key Largo Key	April 2, 1963	1963
FLMNH_024	Key Largo Key	April 2, 1963	1963
FLMNH_025	Key Largo Key	April 2, 1963	1963
FLMNH_026	Key Largo Key	April 2, 1963	1963
FLMNH_027	Key Largo Key	April 2, 1963	1963
FLMNH_028	Key Largo Key	April 2, 1963	1963
FLMNH_029	Key Largo Key	April 1, 1963	1963
FLMNH_030	Key Largo Key	April 1, 1963	1963
FLMNH_031	North Key Largo	March 15, 1979	1979
FLMNH_032	North Key Largo	March 15, 1979	1979
FLMNH_033	North Key Largo	March 15, 1979	1979
FLMNH_034	North Key Largo	March 15, 1979	1979
FLMNH_035	North Key Largo	March 15, 1979	1979
FLMNH_036	North Key Largo	March 15, 1979	1979
FLMNH_037	North Key Largo	March 15, 1979	1979
FLMNH_038	North Key Largo	March 15, 1979	1979
FLMNH_039	North Key Largo	March 15, 1979	1979
FLMNH_040	North Key Largo	March 15, 1979	1979

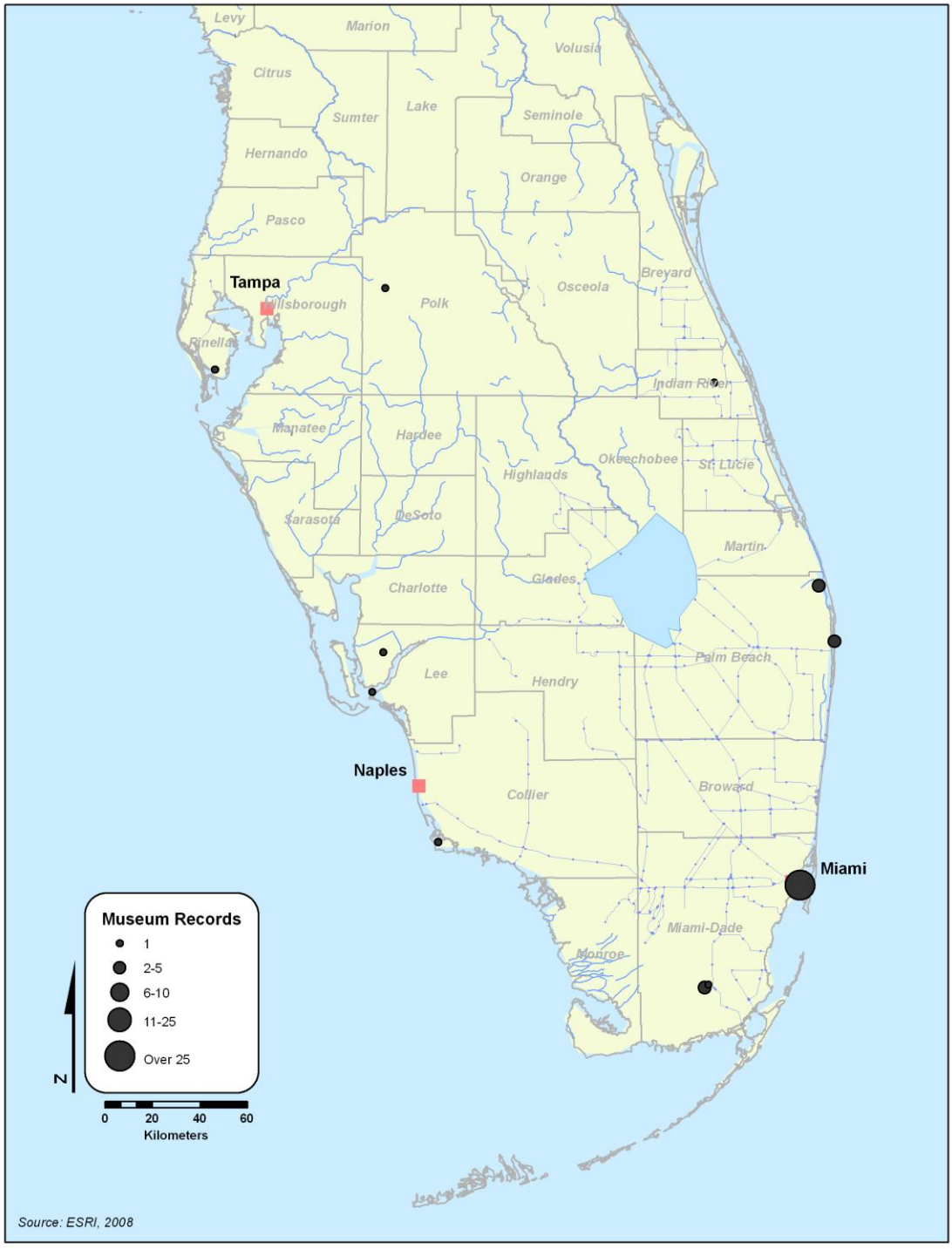


Figure 3-2. Museum specimens of *C. t. bethunebakeri* collected from 1900-1930, the frontier era as per Solecki (2001).

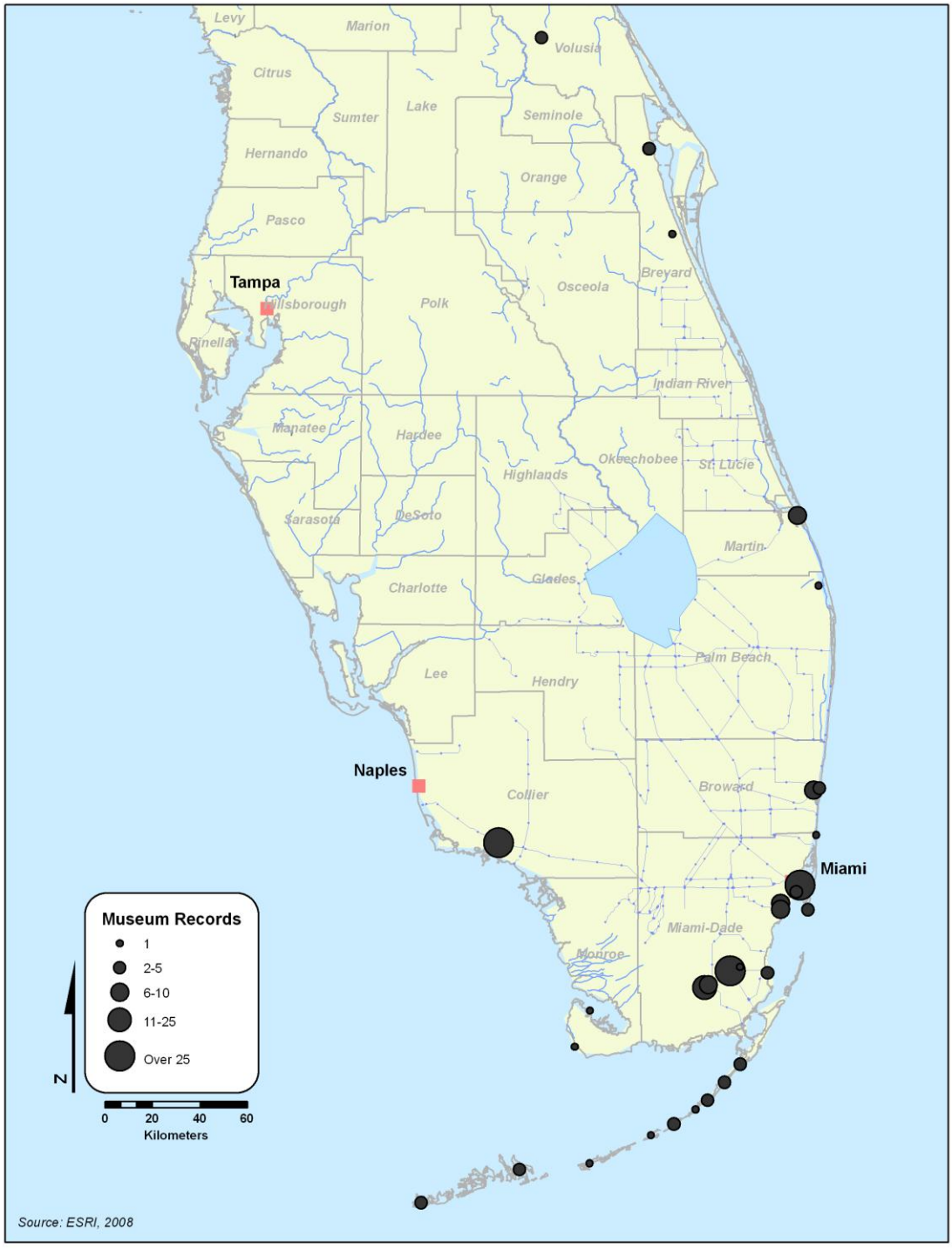


Figure 3-3. Museum specimens of *C. t. bethunebakeri* collected from 1931-1970, the development era as per Solecki (2001).

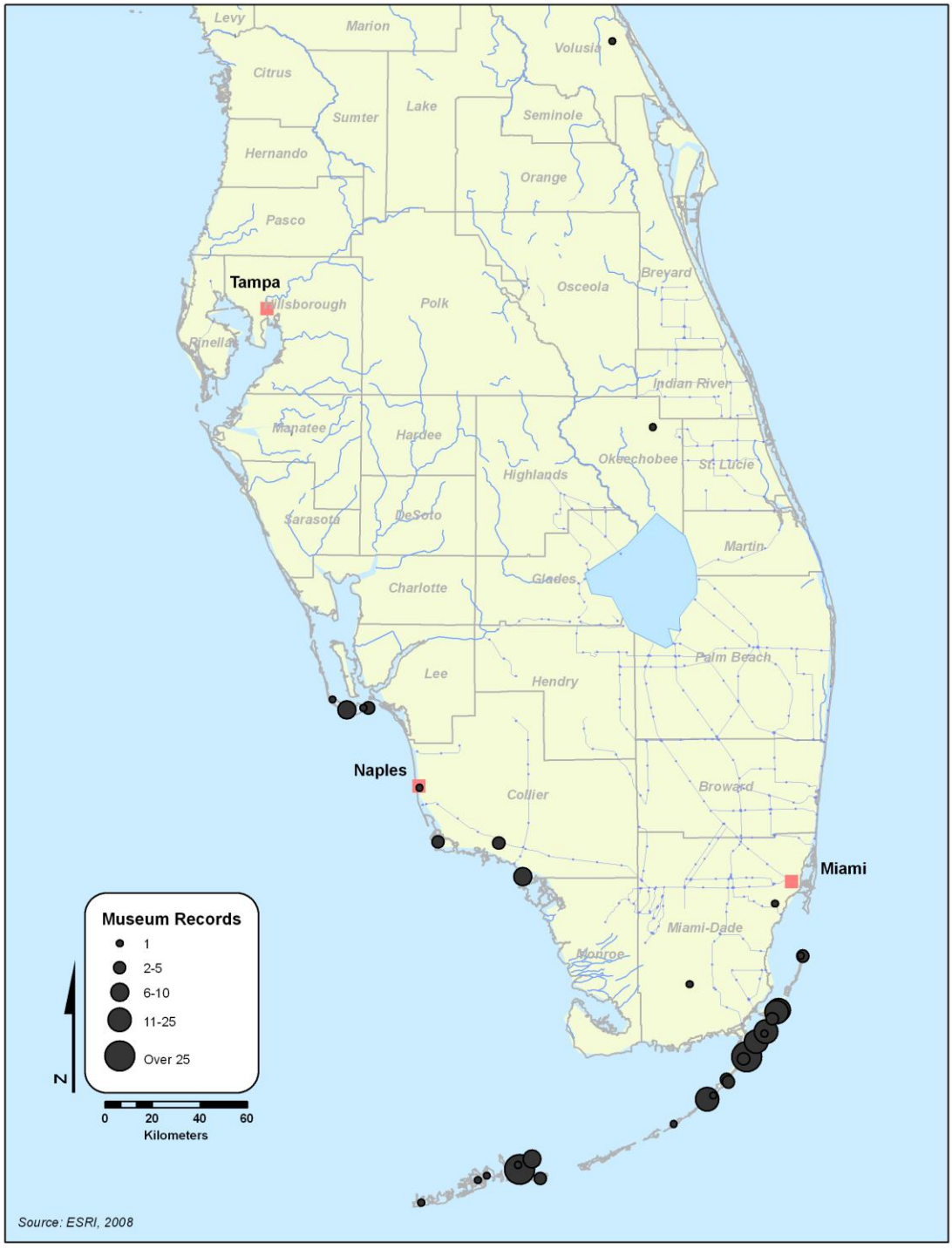


Figure 3-4. Museum specimens of *C. t. bethunebakeri* collected from 1971-present, the globalization era as per Solecki (2001).

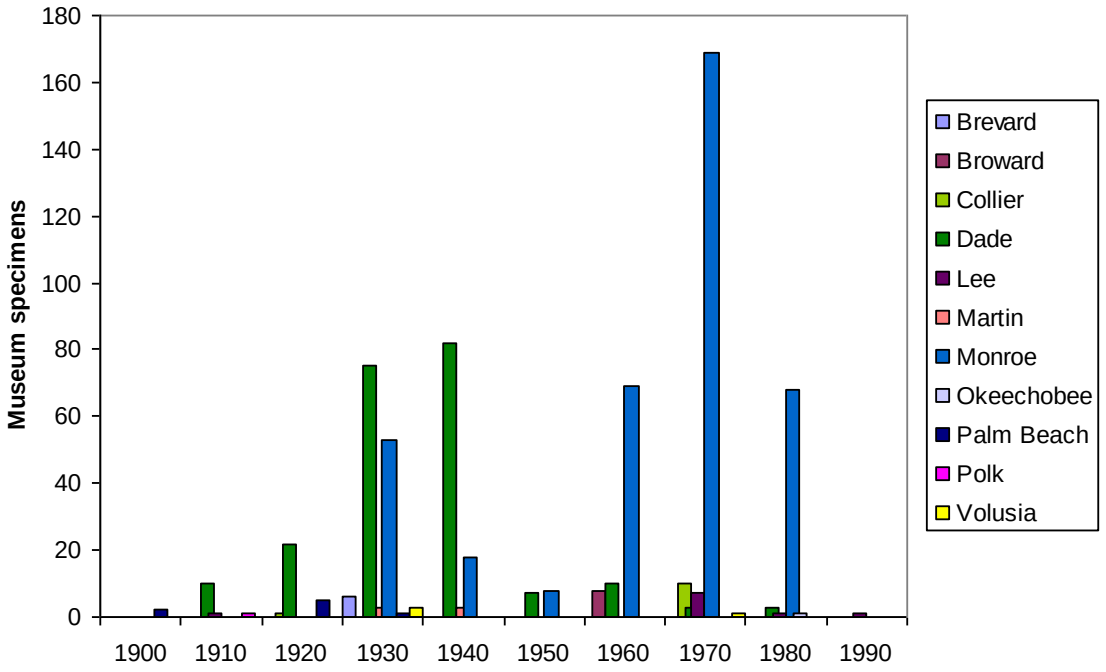


Figure 3-5. Number of *C. t. bethunebakeri* specimens deposited in five major museum collections by Florida County from 1900-1990.

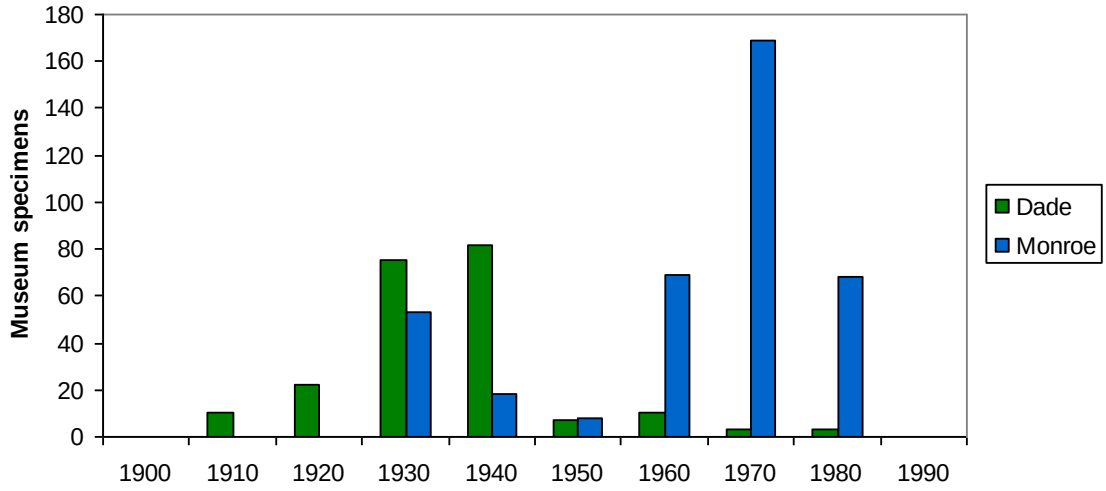


Figure 3-6. Number of *C. t. bethunebakeri* specimens deposited in five major museum collections for Dade and Monroe Counties, Florida.

Table 3-3. Results of ANOVA analyses comparing historical (1927-1988 FLMNH) and contemporary (2002-2006 BHSP) *C. t. bethunebakeri* wing chord lengths (mm mean ± standard error) over several variables. *P*-values are italicized when significant at $P < 0.05$.

Variable	BHSP	BHSP (mm)	FLMNH	FLMNH (mm)	*P*-value
Female	114	11.868 ± 0.077	121	11.440 ± 0.091	*0.00040*
Male	162	11.386 ± 0.069	204	11.212 ± 0.075	0.09800
Wet season	203	11.687 ± 0.059	146	11.273 ± 0.083	*0.00003*
Dry season	34	10.971 ± 0.177	179	11.317 ± 0.082	0.08900
Location (BHSP vs. historical Lower Keys)	276	11.585 ± 0.054	58	11.462 ± 0.141	0.35700
Total	276	11.585 ± 0.054	325	11.297 ± 0.058	*0.00040*

Table 3-4. Overview of molecular diversity measures for *C. t. bethunebakeri* populations at microsatellite locus CthB119. *N*, number of samples with positive PCR results; N_A, number of observed alleles; n_e, effective number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity, and *P*-value of Hardy-Weinberg equilibrium test significance. Tests are assessed for significance after a sequential Bonferroni correction; values not in equilibrium are in italics.

Population	*N*	N_A	n_e	H_O	H_E	*P*-value
Mixed	4	5.000	4.000	0.500	0.857	0.075
Key Largo 1940s	6	4.000	2.769	0.500	0.697	0.416
Key Largo 1963	8	5.000	3.459	0.500	0.759	0.126
North Key Largo 1979	6	4.000	3.789	0.667	0.803	0.043
BHSP Sept. 2005	24	4.000	1.417	0.083	0.301	*0.000*
BHSP June 2006	36	7.000	1.427	0.162	0.275	*0.002*
KWNWR Feb. 2008	16	4.000	2.798	0.250	0.663	*0.000*

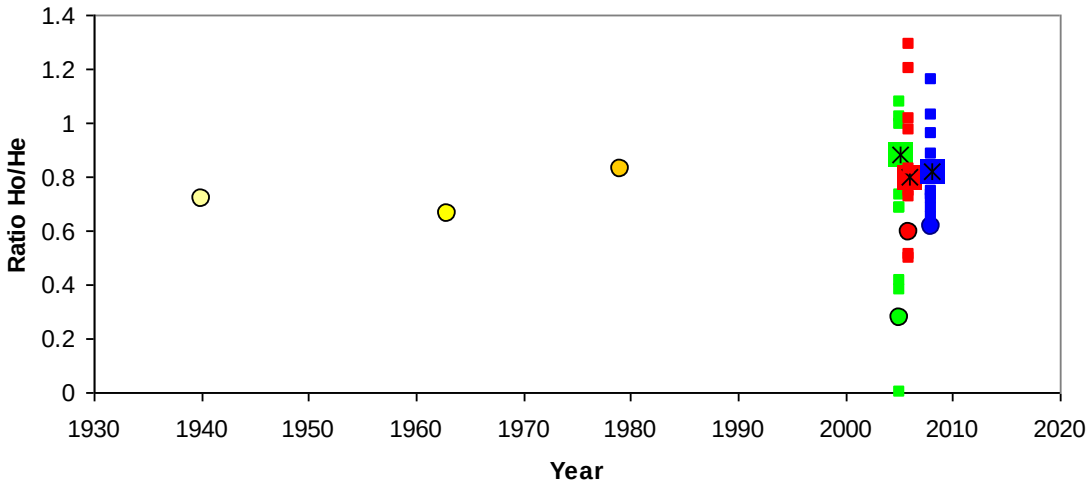


Figure 3-7. Ratio of H_O/H_E values for historical Key Largo (yellow) and contemporary BHSP (green and red) and KWNWR (blue) populations of *C. t. bethunebakeri*. Circled points are from locus CthB119. In contemporary populations, squares represent the remaining 11 individual microsatellite loci and the average ratio per year (across all 12 loci) are shown as a square with an asterisk.

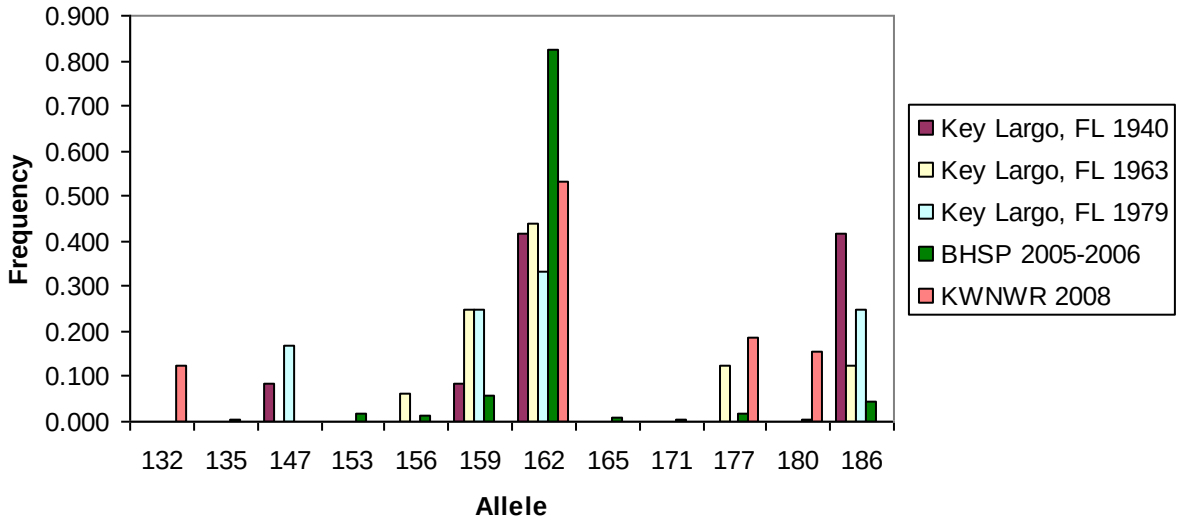


Figure 3-8. Frequency of alleles at microsatellite locus CthB119 in all *C. t. bethunebakeri* populations. Data are from museum samples from Key Largo, Florida and current samples from BHSP, Bahia Honda State Park, Florida.

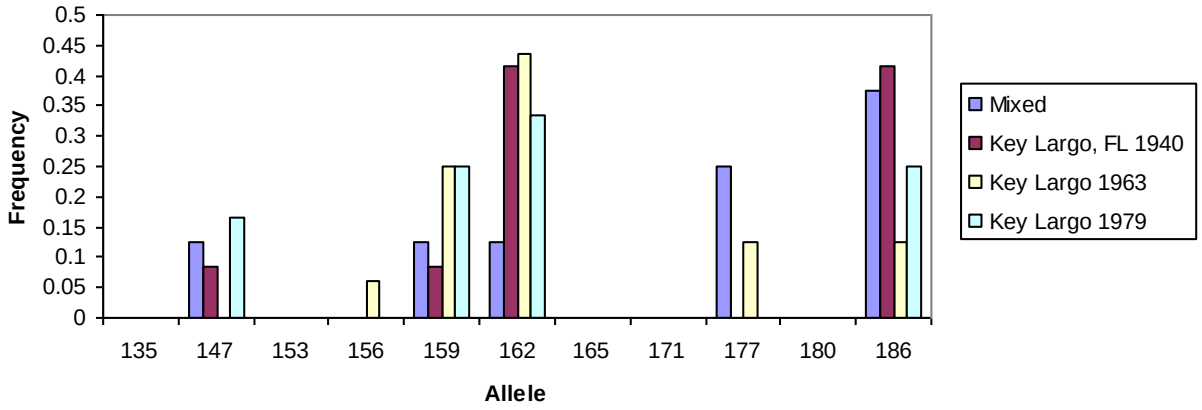


Figure 3-9. Allelic frequencies at microsatellite locus CthB119 of historical populations of *C. t. bethunebakeri*.

Table 3-5. Frequency of alleles at microsatellite locus CthB119 in historical and current populations of *C. t. bethunebakeri*. Allele, size in base pairs; Populations (*N*) of Mixed Florida origin; KL, Key Largo, Florida; BHSP, Bahia Honda State Park, Florida, and KWNWR, Key West National Wildlife Refuge, Florida. A single historical allele is in italics.

Allele	Mixed (4)	KL 1940s (6)	KL 1963 (8)	North KL 1979 (6)	BHSP 2005 (24)	BHSP 2006 (36)	KWNWR 2008 (16)
132	0.000	0.000	0.000	0.000	0.000	0.000	0.125
135	0.000	0.000	0.000	0.000	0.000	0.014	0.000
147	0.125	0.083	0.000	0.167	0.000	0.000	0.000
153	0.000	0.000	0.000	0.000	0.083	0.000	0.000
156	0.000	0.000	0.063	0.000	0.000	0.028	0.042
159	0.125	0.083	0.250	0.250	0.063	0.056	0.063
162	0.125	0.417	0.438	0.333	0.833	0.833	0.583
165	0.000	0.000	0.000	0.000	0.000	0.014	0.000
171	0.000	0.000	0.000	0.000	0.000	0.014	0.000
177	0.250	0.000	0.125	0.000	0.000	0.000	0.229
180	0.000	0.000	0.000	0.000	0.021	0.042	0.042
186	0.375	0.417	0.125	0.250	0.000	0.000	0.042

Table 3-6. Pairwise population differentiation between each of the historical groups and contemporary populations of *C. t. bethunebakeri*. F_{ST} values (above the diagonal) and R_{ST} values (below the diagonal) with respective levels of significance; *$P < 0.05$, **$P < 0.001$.

	Mixed	1940	1963	1979	BHSP	KWNWR
Mixed		0.000	0.000	0.000	0.420**	0.039
1940	0.000		0.000	0.000	0.287**	0.033
1963	0.000	0.000		0.000	0.350**	0.000
1979	0.000	0.000	0.000		0.378**	0.004
BHSP	0.059	0.216*	0.542**	0.535**		0.305**
KWNWR	0.006	0.000	0.000	0.000	0.462**	

CHAPTER 4
GENETICS OF WILD POPULATIONS

Introduction

The Miami blue butterfly (*Cyclargus thomasi bethunebakeri*) is a small, brightly colored butterfly endemic to southern Florida with two subspecies occurring in the Bahamas and several species found throughout the Caribbean (Smith et al. 1994). This taxon has received considerable attention in the mainstream media due to its charismatic name and history. Once widespread and locally abundant in the south Florida mainland, *C. t. bethunebakeri* has been eliminated from much of its former range due to ever-expanding urbanization and the associated loss of coastal habitat (Calhoun et al. 2002). Verified observations rapidly declined through the 20th century, and by the early 1990s *C. t. bethunebakeri* was presumed to be extirpated. Fortunately, the taxon was rediscovered on 29 November 1999 in a small population of less than 100 individuals within the boundaries of Bahia Honda State Park (BHSP) in the Lower Florida Keys (Ruffin & Glassberg 2000). In December 2007, a second colony of *C. t. bethunebakeri* was discovered on the Marquesas Islands in Key West National Wildlife Refuge (KWNWR), 50 km west of Key West (Cannon 2007).

The two remaining populations are subject to an increased threat of extirpation from a variety of environmental and human factors including hurricanes, drought, predation, illegal collecting, loss of genetic diversity, habitat alteration, and mosquito control practices. The BHSP population and the taxon as a whole are therefore in an extremely tenuous situation. There exist no known additional neighboring populations capable of re-colonizing habitat areas should they be impacted. The BHSP and KWNWR populations are over 70 km apart, much of which is over open water, and it is unlikely that such a small butterfly could travel this distance in its short adult life span of less than a week (FWC 2003). The resulting low census numbers and

population vulnerability make *C. t. bethunebakeri* one of the most critically imperiled insects in the United States.

Reduced genetic diversity and reduction of habitat area are two potentially influential factors leading to species extinction (Avise 1996). It has been proposed that demographic factors may affect endangered species before the genetics and deleterious effects of inbreeding even have a chance to take effect (Lande 1995), although other studies have shown that genetic factors impact the rate of extinction of endangered populations (Newman & Pilson 1997; Saccheri et al. 1998; Spielman et al. 2004). In this study, I determine and compare the levels of genetic diversity in both the BHSP and KWNWR populations and look for evidence of a genetic bottleneck. I also assess how diversity changes over time within the BHSP population. Lastly, I examine gene flow between the habitat patches (subpopulations) of the BHSP metapopulation. These efforts will be useful in conservation efforts focused on this taxon as well as other endangered butterfly taxa of the region.

I have selected several time scales over which to assess genetic diversity in the BHSP population. I have done so to 1) increase statistical power by increasing sample sizes when samples can be pooled according to biologically-sound reasoning (as recommended by Frankham et al. 2003) and to 2) highlight any fine-scale changes in molecular diversity that may occur throughout seasonal changes and with the flux of population numbers. The BHSP population exists as a metapopulation, with butterflies and larvae occupying some but not all suitable habitat patches on the island. The level of gene flow and connectivity between discrete habitat patches is currently unknown. I therefore conducted analyses of BHSP at several spatial scales to uncover the different levels of gene flow. I analyzed BHSP as a single population (with no subdivision), as individual colonies occupying discrete habitat patches (as several

groups acting in a metapopulation structure), and as a division of clumped colonies versus other, more spatially-distant colonies. In this manner, I hope to analyze genetic measures at all biologically-relevant time and spatial scales.

I used traditional genetic diversity measures and population-level statistics to characterize the genetic diversity of the taxon. Loss of heterozygosity over time may indicate that a population is undergoing inbreeding or otherwise losing evolutionary potential (Frankham et al. 2003). I also completed testing of Hardy-Weinberg equilibrium (HWE), linkage disequilibrium, gene flow (F_{ST} and R_{ST}), and effective population size (N_e). Wright (1978) suggested guidelines for interpreting F_{ST} values, but F_{ST} values vary considerably among taxonomic groups and studies (review in Frankham et al. 2003). Inbreeding coefficients are also calculated according to methods of Weir & Cockerham (1984) to compare relative levels of inbreeding between patches and populations.

Methods

Habitat Maps

I took Global Positioning System (GPS) coordinates (WGS 1984 projection) and notes of habitat condition, quality, and extent of the locations of *C. t. bethunebakeri* colonies in Bahia Honda State Park (BHSP), Florida in September 2005 and April-October 2006. I transformed these data into a geodatabase using ArcGIS 9.2, Environmental Systems Research Institute Redlands – ESRI, Inc., Redlands, California). The point locations were then overlaid on a satellite photo of BHSP taken in 2005 obtained through the Florida Geographic Data Library (http://www.fgdl.org/). I then digitally drew habitat extent onto fine-scale aerial maps by referencing my field notes (Figure 4-1).

Sampling

I developed a relatively non-invasive method to assess molecular diversity in the Bahia Honda State Park (BHSP) and Key West National Wildlife Refuge (KWNWR) populations. I gently captured butterflies in a net and removed a 2 mm^2 wing fragment from the hind wing with small forceps (Figure 4-2). Care was taken to clean forceps to prevent cross-contamination between samples. The removal of such a small amount of wing material did not adversely affect butterfly behavior and butterflies were observed courting, mating, and feeding shortly after the removal of wing fragment. Wing tissue was preserved in 90-100% ethanol. Sample vials were kept cool and in the dark until they were returned to the laboratory -80℃ freezer for storage.

Wing fragments for molecular analyses from the BHSP population of butterflies were sampled from September 2005 thru October 2006 (N = 114) and represent multiple generations of this population (Table 4-1). Individual months represent temporally-discrete generations as these butterflies were sampled over a five-day period in each month. The September 2005 samples were the only representation of BHSP in 2005. In 2006, data were pooled for samples taken in concurrent months (April and May, June and July, and September and October) to increase sample size and the power of statistical analyses. Only data from concurrent months were pooled as this represents a biologically probable breeding unit for this taxon that has a new generation approximately every 30-40 days (FWC 2003). Annual data were pooled to show overall changes in diversity between years. All data from April 2006-October 2006 were pooled for 2006 results.

The KWNWR population was sampled once in February 2008 and represents a discrete breeding population. Two samples were taken from Boca Grande Key and 27 samples were taken from Marquesas Key. Owing to the small sample size from Boca Grande Key, only samples from the Marquesas Keys were considered for genetic analysis of KWNWR.

DNA Extractions and Microsatellite Amplification

I extracted DNA from 2 mm^2 wing fragments using Qiagen DNeasy Blood and Tissue Kits (Qiagen©, USA). I followed the manufacturer's protocols for kit extractions with minor modifications owing to the small size of the wing material I was extracting from (Appendix A). I stored DNA extractions at -20℃ until I proceeded with PCR amplifications of the twelve polymorphic microsatellite loci. All PCR reactions follow the protocols outlined in Saarinen et al. (2009). Between 5-10% of all BHSP samples were re-genotyped at nine microsatellite loci to determine genotyping error rates for each locus. All private alleles were individually assessed for validity and that they were not the result of genotyping or scoring error.

Statistical Analyses

Microsatellite data were organized in Microsoft Excel and formatted using Convert (Glaubitz 2004) to be used in other software packages. Micro-Checker version 2.2.3 (van Oosterhout et al. 2004) was used to test for the presence of null alleles as problems of allelic drop-out have been identified for PCR-amplified products in Lepidopteran microsatellites (Zhang 2004) as well as for studies utilizing noninvasive genetic samples of low quantity (Taberlet et al. 1999). I used Arlequin version 3.01 (Excoffier et al. 2005) to estimate the observed and expected heterozygosities (H_O and H_E) and Hardy-Weinberg equilibrium was tested for with an exact test using a Markov chain with 1,000,000 forecasted chain length and 100,000 dememorization (burn-in) steps. Hardy-Weinberg equilibrium significances were evaluated after a sequential Bonferroni correction (Rice 1988). I used FSTAT version 2.9.3.2 to test for linkage disequilibrium (Goudet 2001) and confirmed results with GDA 1.1 (Lewis & Zaykin 2001). I used GenAlEx 6.1 (Peakall & Smouse 2006) to estimate the effective number of alleles per locus and analyses of molecular variance across generational samples. I used FSTAT to calculate allelic richness, a measure of the number of alleles independent of sample size

(Goudet 2001). The independence from sample size allows for the comparison of allelic richness values between generations or populations of varying size. *F*-statistics, R_{ST} values, and inbreeding coefficients were calculated in GenAlEx 6.1 (Peakall & Smouse 2006). Both F_{ST} and R_{ST} values were calculated for estimates of gene flow as each measure is based on a different assumption of marker mutation rate. R_{ST} has been recommended for microsatellite data as it follows a Stepwise Mutation Model (SMM) and may detect more population differentiation than F_{ST} (Slatkin 1995). F_{ST} (Wright 1965) uses allele identity rather than size corresponding to the Infinite Alleles Model (IAM) to measure population subdivision. Both F_{ST} and R_{ST} were calculated and tested for significance over 9,999 permutations. Sequential Bonferroni corrections were performed on all data with multiple comparisons to correct for Type 1 error (Rice 1988). The program Bottleneck (Cornuet & Luikart 1996) was used to look for genetic signatures typical of a population that has undergone a bottleneck. The SMM, IAM, and TPM (Two-Phase Model) of microsatellite evolution were calculated and 1000 iterations were run for each model. For the latter model, I set the proportion of SMM at 70% and ran allowing 30% variance for TPM. I tested individual, discrete breeding populations (i.e. those samples collected within the same week ensuring no overlap of generations) that met the sample size criterion of 15-40 individuals (Cornuet & Luikart 1996). To ensure that the program worked with my data, I also tested a known population bottleneck using data from the captive breeding colony of *C. t. bethunebakeri* housed at the University of Florida. The October 2006 captive colony (CC-10-2006) had been allowed to breed without the introduction of new genetic material for three-four generations, thus representing a bottlenecked population. I used the program NeEstimator (Peel et al. 2004) to estimate the effective population size (N_E) of the BHSP population. This program uses a moments-based temporal model (Waples 1989) to estimate the effective population size

over time. For these analyses, consecutive generations were required to estimate effective population sizes. Lastly, I used the program Structure 2.0 (Pritchard et al. 2000) to look for genetic structure across generations and in the two *C. t. bethunebakeri* populations. This program assumes no specific mutation model and is appropriate for unlinked microsatellite data (Pritchard et al. 2007). I examined all allelic data from BHSP (N = 114) for population structuring across generations. I then looked for population differentiation between BHSP and KWNWR by analyzing the September 2005 BHSP population and the February 2008 KWNWR population. The September 2005 BHSP sample was chosen for comparison with the KWNWR population as both populations were similar in sample size. I used the population admixture model implemented in Structure with correlated allele frequencies among populations as recommended by Falush et al. (2003) for situations with subtle population structure. No prior population information was included in these analyses. I ran 5 replicates at each estimated population number (K = 1 to K = 5) with a burn-in of 50,000 iterations and collection of data over 500,000 iterations. Exploratory testing using the no admixture model showed similar results to the admixture model. The September 2005 BHSP and the February 2008 KWNWR populations were also run with these model parameters additionally allowing prior population information to assist with clustering.

Estimation of Microsatellite Mutation Rate

My goal in investigating the mutation rate of the microsatellite markers employed in this study was to determine whether a high mutation rate was responsible for observed genetic diversity. Microsatellite mutation rates have been measured across taxa with rates ranging from 10^{-6} in *Drosophila* mutation accumulation lines (Schug et al. 1998) to 10^{-3} in Gastropoda (Gow et al. 2005) and Insecta (Crozier et al. 1999). Extensive microsatellite surveys of the human genome, utilizing over 5,000 microsatellites revealed a mutation rate of ~10^{-4} (Weissenbach et al.

1992; Dib et al. 1996; Whittaker et al. 2003). A recent, extensive survey of the wheat genome using a gene genealogy approach showed a mean mutation rate of 1.68×10^{-3} across 21 microsatellite markers when experimental wheat populations were tracked over multiple generations (Raquin et al. 2008). My goal was to determine whether the microsatellite markers used in this study fell within this range of mutation rates, or whether an elevated rate of microsatellite mutation exists and was responsible for an inflated value of molecular diversity. To determine the overall mutation rate of the 12 microsatellite markers used in this study, I genotyped two parents and 21 of their offspring. This family line was raised apart from other individuals so that there would be no mixing between family groups that could lead to erroneous results. By genotyping two parents and 21 offspring at 12 loci, I achieved 504 comparisons in which to look for mutations. As microsatellites are passed on by Mendelian inheritance, any change in allele size from parent to offspring can be considered a mutation. I manually confirmed all genotyping results within the program GeneMapper version 4.0 (ABI) and re-genotyped any results that yielded a mutation three times to confirm results were not a result of scoring error.

Results

Molecular Diversity

Tests for linkage disequilibrium of the 12 microsatellite markers showed that loci were unlinked (after a sequential Bonferroni correction). Therefore, the data represented here are from 12 independent loci in the *C. t. bethunebakeri* genome. Null alleles were inferred at seven loci (CthB101, CthB106, CthB115, CthB117, CthB119, CthC12, and CthC116) due to the presence of homozygote excess. Micro-Checker (van Oosterhout et al. 2004) showed no evidence of large allele drop-out or scoring error due to stuttering. I further verified genotyping results by re-genotyping 5-10% of samples at nine loci (Table 4-2). Samples were accurately re-

genotyped at loci between 58.33-100.00%. In loci where successful re-genotyping rates were less than 100%, samples were re-genotyped a third time to verify results. All statistical analyses were performed on this corrected dataset.

My first objective was to assess the molecular diversity within the Bahia Honda State Park (BHSP) population. The effective number of alleles (n_e) in the BHSP remained relatively constant over time, at both a monthly (generational) and annual scale (Table 4-3). Allelic richness was also stable over time in BHSP, with values ranging from 2.988-3.121 when averaged across the 12 microsatellite loci from September 2005 – October 2006. Allelic richness in KWNWR was 3.790 in February 2008, higher than BHSP values. There were seven loci in Hardy-Weinberg equilibrium (HWE) in each month sampled, but the individual loci in HWE differed between months (Table 4-4). Locus CthC124 was monomorphic in all BHSP generations except the June 2006 population (when it was in HWE). The population on KWNWR is in HWE at 10 of 12 loci.

Mean inbreeding coefficients were estimated across 12 microsatellite loci for each sampled group (subpopulation) over time (Figure 4-3). These values are equivalent to the *f* of Weir & Cockerham (1984) as they were calculated on single populations from allelic frequency data. I calculated values in this manner to assess inbreeding within individual subpopulations. The most negative value (-0.289, SE 0.076) was found in the Buttonwood 2006 group. The most positive values (0.285, SE 0.146), Bayside 2005, may denote inbreeding or undetected null alleles, both likely options given the nature of the subpopulation and the markers used to study them. Most subpopulations analyzed (three of 11) showed a positive *f* value, denoting the possibility of inbreeding or null alleles, although individual subpopulation *f*-values did not vary greatly over time. F_{IS} values measure the heterozygosity reduction in an individual relative to its

subpopulation, and these values were consistent for the 2005 and 2006 BHSP populations; F_{IS}=0.112 (SE 0.104) and F_{IS}=0.120 (SE 0.070), respectively.

Results from the program Bottleneck (Cornuet & Luikart 1996) do not show significant evidence of a genetic bottleneck in any of the BHSP generations analyzed (Table 4-5). The Wilcoxon signed rank tests (Luikart & Cornuet 1997) for the September 2005, May 2006, and June 2006 samples failed to show excess heterozygosity under the three models (IAM, TPM, and SMM). Additionally, these three BHSP generations showed an L-shaped allele frequency distribution that is characteristic of a population in mutation-drift equilibrium. Populations that have undergone a bottleneck show a mode shift in their allelic distribution. Only the captive colony population (CC-10-2006) that had undergone a known bottleneck showed evidence of a mode shift. This population also showed heterozygosity excess under the IAM model. The KWNWR population is the only other population to show heterozygote excess (also under the IAM, P = 0.003) but showed no evidence of a mode shift. Evidence of heterozygote deficiency was significant under the SMM in all BHSP populations. The CC-7-2006 population showed deficiency under the TPM and the SMM (Table 4-5).

Effective population sizes of the Bahia Honda State Park population were evaluated using the program NeEstimator (Peel et al. 2004). Results were generated by comparing allelic frequencies in consecutive generations in a moments-based temporal model. These data are summarized in Table 4-6 and show an estimate of 26.8 (12.5-68.8, 95% CI) for the effective population size between May 2006 to October 2006 for the BHSP population. Estimates could not be calculated for the KWNWR population as I have measurements from only one generation.

Gene Flow in Bahia Honda State Park

Analyses of microsatellite frequencies were also used to assess gene flow between habitat patches (Figures 4-4 and 4-5). No values were computed when only one sample was available in

a subpopulation (no values are given for the Buttonwood population in 2005). When considering individual subpopulations on the South end of BHSP, only minor population subdivision is observed, with most pairwise values between neighboring segments at or very near 0.000. No F_{ST} or R_{ST} values are significant in 2005, but they are significant in 2006 (Figure 4-4). F_{ST} and R_{ST} values on the South end are higher in 2006 than in 2005, possibly due to the absence of individuals from the Bridge population. More population subdivision is evident when considering the island of BHSP as a whole (Figure 4-5). The Buttonwood and Sandspur subpopulations are well linked by the Main Road population (F_{ST} and R_{ST} effectively 0.000). The South end colonies show some division from the other populations (Buttonwood, Main Road, and Sandspur). Again, many significance values are above $P = 0.05$ when comparing levels of gene flow. Interestingly, the smallest of these differences is between the South end colonies and Sandspur, which is the farthest colony by geographic distance. These values still showed relatively little differentiation between subpopulations. Analyses of AMOVA showed high levels of variation among individuals in both 2005 and 2006. In 2005, 77% of molecular variance was among individuals, 23% within individuals, and 0% among populations. In 2006, 77% was among individuals, 20% within individuals, and 3% among populations when individual habitat patch populations were considered for each year.

Analysis of all BHSP allelic data using the program Structure (Pritchard et al. 2000) is summarized in Table 4-7. The highest Ln $P(X/K)$ is for $K = 2$ (after 5 independent runs), suggesting that the BHSP data are derived from two source populations. Further analyses of the data show no structuring due to time of collection, sex, or location. The two populations show significant separation, but no immediate explanation for this division of BHSP data is evident (Figure 4-6).

Estimations of genetic differentiation between BHSP and KWNWR (utilizing BHSP September 2005 and KWNWR February 2008 datasets, respectively) yielded F_{ST} = 0.191 (P = 0.0001) and R_{ST} = 0.275 (P = 0.0001) after 9,999 permutations. Analysis from Structure 2.0 (Pritchard et al. 2000) showed that the estimated log-likelihood values increased with increasing K (Table 4-8). This situation is described in Evanno et al. (2005) as a problem with the program Structure where likelihood values may increase with increasing K, and continue to do so after the "real" K has been reached. Additional analyses using the same model parameters but including prior population information about collection origin (BHSP or KWNWR) show the highest Ln P(X/K) is for K = 2 (Table 4-8) and show a clear distinction between the KWNWR and BHSP populations (Figure 4-7).

Mutation Rate of Microsatellites

All private alleles fit the expected mutation pattern and the 504 comparisons of microsatellites revealed one mutation at one locus. CthB103, a locus with a trinucleotide repeat, an offspring had an allele at 162 bp, when its parents' genotypes were 153, 233 and 153, 153. This mutation was therefore not generated in a step-wise manner. It is unknowable whether the mutation occurred from the allele expanding from 153 to 162 bp or whether it shrank from 233 to 162 bp. This single mutation leads to a mutation rate of 1.98×10^{-3}. This result is very comparable to the extensive survey of the wheat genome showing an average rate of 1.68×10^{-3} (Raquin et al. 2008). At the mutation rate of the wheat genome, a mutation is expected every 595 comparisons on the average, and every 201 comparisons at the high end (4.97×10^{-3}).

Discussion

Overall patterns of genetic diversity in the BHSP populations (mean overall observed heterozygosity of 39.5%) are similar to or slightly lower than other non-migratory butterfly species' studies utilizing microsatellite markers. Butterfly species that are not considered

endangered show a range of 40.97-48.37% mean overall observed heterozygosity (Keyghobadi et al. 2002; Fauvelot et al. 2006; Sarhan 2006). The federally-endangered Karner blue butterfly (*Lycaeides melissa samuelis*) exhibits 28.25% mean observed heterozygosity (Anthony et al. 2001) but the endangered *Polyommatus bellargus* registers a mean observed heterozygosity value higher than non-threatened species, 53.2% (Harper et al. 2000). Perhaps concordantly, diversity in *C. t. bethunebakeri* was higher in the KWNWR population than the BHSP population (mean observed heterozygosity of 51% versus 39.5%). Allelic richness was also higher in KWNWR, and the KWNWR population may represent an important source of variation to be considered for future conservation efforts for this taxon.

Heterozygosity may still be underestimated at some *C. t. bethunebakeri* microsatellite loci due to the presence of allelic dropout, even after my efforts to correct for this with re-genotyping. This underestimation has multiple implications, such as the under-representation of the number of loci in Hardy-Weinberg equilibrium. Furthermore, as the program Bottleneck (Cornuet & Luikart 1996) uses the signal of heterozygote excess to interpret a population bottleneck, there may have been a population bottleneck that was undetected due to genotyping error. This shows the importance of re-genotyping to ensure that results are correctly interpreted, as denoted by Pompanon et al. (2005). The validation of genotypic data is especially important in Lepidoptera, as this group shows a high rate of PCR failure due to changes in flanking sequences (Zhang 2004). Because I re-ran PCRs and re-genotyped a percentage of samples that showed discord between genotyping scores, I am confident in the results I present. Lastly, the program Micro-Checker detected null alleles at seven loci due to homozygote excess. These may not be null alleles but rather representative of the reduced diversity of the population.

Most researchers have assumed that the BHSP population has undergone a severe population bottleneck (FWC 2003) but the data presented here show no signature of a bottleneck. It may be more likely that this *C. t. bethunebakeri* population has been subjected to a more *historic* bottleneck, one that is not detectable by my methods. Only recent bottlenecks, within 4 N_E generations, are able to be detected by the program Bottleneck (Cornuet & Luikart 1996). If the effective population size is approximately 27 (as per NeEstimator results), then a bottleneck could only be detected if it occurred within 108 generations. As the *C. t. bethunebakeri* generation time is short (one generation per 30-40 days) it is likely that the population could have undergone a bottleneck in the 1980s and 1990s when populations were in severe decline (Calhoun et al. 2002), and then allelic levels returned to mutation-drift equilibrium in the following months and years. The only wild population to show a sign of bottleneck is from KWNWR, which may be a more recently founded population. The new colonization of this area would certainly represent a bottleneck event, and confirms the ability of the program Bottleneck to detect such an event.

The utility of this program is further confirmed as it has verified the bottleneck event that occurred in the captive colony of butterflies in October 2006. Wild butterfly stock had last been incorporated into the captive colony in June 2006, thus, four generations had elapsed with no new "migrants" into the captive population (J.C. Daniels, personal communication). The detection of a bottleneck at this juncture highlights the importance of infusing captive colonies with wild genetic stock on a regular basis. The detection of all bottlenecks in this study was only significant under the IAM, denoting that the TPM and SMM are poor at detecting past bottlenecks in these populations. Similar results were found in the Adonis blue butterfly *Polyommatus bellargus* (Harper et al. 2003), highlighting the need to utilize all mutation models

so as not to overlook significant results. Additionally, my analysis of microsatellite mutations in this taxon revealed a mutation event that did not occur in a stepwise manner. The SMM was most successful at detecting heterozygote deficiency, which is of great importance for such small, endangered populations, and which was detected in all BHSP populations analyzed.

Even with the increased sampling from 2005 vs. 2006 (N = 24 vs. N = 77) there was minimal increase in the effective number of alleles (n_e) in the BHSP population. This confirms that my sampling practices for both years have effectively captured the diversity present in the population. These data show that the BHSP population has retained an adequate amount of genetic diversity to maintain the population despite perceived changes in overall population size. Census estimates (from daily transect counts) for 2005 are 5-133 individuals and 15-82 individuals for 2006 (J.C. Daniels, unpublished data). These data roughly agree with results found for effective population size based on genetic data which show the population supporting approximately 27 butterflies (13-70 individuals, 95% CI).

Inbreeding coefficients (f) in individual habitat patches on BHSP and KWNWR vary greatly over space and time, from f = -0.289-0.285. The observation that inbreeding coefficients in discrete habitat patches (population segments) change over time may speak more to habitat quality and the ability to disperse to better habitat. The inbreeding coefficient of the common but poorly-dispersing lycaenid *Arhopala epimuta* is comparatively very high (denoting high levels of inbreeding), ranging from f = 0.407-0.503 (Fauvelot et al. 2006). Inbreeding coefficients in the non-endangered, high-dispersing butterfly *Heliconius charitonia* in the Miami area are all negative (Kronforst & Fleming 2001). The overall F_{IS} values, however, of BHSP *C. t. bethunebakeri* are consistent and relatively close to 0 and are similar to the swallowtail butterfly *Papilio machaon* (F_{IS} = 0.062) (Hoole et al. 1999). In both *C. t. bethunebakeri* and the

swallowtail butterfly, high gene flow is evident, and the F_{IS} value indicates neither an excess nor deficit of heterozygotes. The overall message appears to be that *C. t. bethunebakeri* is not particularly inbred in the wild and values are comparable to those of other butterflies with varying dispersal abilities and population numbers. Care should still be taken in interpreting genetic results as several loci are still not in HWE on a regular basis and there appears to be a consistent heterozygote deficiency in the BHSP population. The population needs to be continuously monitored for drops in allelic diversity and changes in frequencies across loci. Such measurements offer a yardstick for overall population health.

The low F_{ST} and R_{ST} values evaluated in this study demonstrate the high levels of gene flow between subpopulations (habitat patches) on BHSP. Such values are explained by either high levels of gene flow or a recent colonization or expansion event on BHSP. As this population was discovered in 1999, over 70 generations have been completed, allowing sufficient time for allelic values to diverge after a founder event. This reinforces the hypothesis that low F_{ST} and R_{ST} values are due to high levels of gene flow between patches.

In 2005, no significant differences were observed between habitat patches on the South end of BHSP (Figure 4-4A). Within the South end of BHSP, it appears that small habitat patches (like the Bridge patch) may be crucial in providing links between the other subpopulations in the area. When the Bridge patch was of poor quality and few butterflies resided there in 2006, estimates of population differentiation between the upper and lower walkway and Bayside areas increased and were significantly different (Figure 4-4B). The Main Road population similarly represents an important link between South end and Sandspur populations (Figure 4-5). The South end and Buttonwood populations, however, showed the highest F_{ST} and R_{ST} values (0.051 and 0.276, respectively), with significant values in the latter measure. These populations are

close in terms of geographic distance, but there may be habitat or demographic barriers to gene flow. The overall estimate of F_{ST} in another endangered lycaenid butterfly, *Polyommatus bellargus,* is 0.127 in populations separated by much greater distance (Harper et al. 2003). Results from the Structure analysis showed two distinct populations on BHSP, but they were not structured by sex, time, or location. This suggests that there is an internal rescue occurring on BHSP and that gene flow between source patches may effectively rescue dwindling subpopulations. Due to genetic mixing, exact patterns were not evident from the Structure results. Furthermore, data showing high gene flow and low levels of inbreeding in close habitat patches should be carefully considered when partaking in reintroduction exercises and conservation planning for *C. t. bethunebakeri* and taxa in similar metapopulation structures. Additional analysis of the KWNWR population may uncover more metapopulation structuring in this region with future sampling and monitoring endeavors.

The comparison of the BHSP and KWNWR populations (September 2005 and February 2008, respectively) shows $F_{ST} = 0.191$ ($P = 0.0001$) and $R_{ST} = 0.275$ ($P = 0.0001$). This amount of population differentiation is perhaps lower than would be expected for populations that are over 70 km apart. This division is corroborated by results produced by Structure, showing the division between the BHSP and KWNWR populations. These values suggest the hypothesis that separation between the populations was recent. Populations between KWNWR and BHSP once existed, linking the two populations, and the recent absence of these populations has broken the gene flow. Analysis of COI mitochondrial sequence data between individuals from BHSP and several islands of KWNWR show identical nucleotide sequences, lending further evidence that these butterflies are the same taxon (E.V. Saarinen, unpublished data.). Divergence in microsatellite allele frequency has not yet extended to nucleotide changes. Further confounding

this story, however, is the fact that larvae of KWNWR feed on host plants of a different family from those of BHSP. Larvae on KWNWR feed on *Pithecellobium spp.* (Fabaceae) and BHSP larvae feed exclusively on *Caesalpinia bonduc* (Fabaceae), even though *Pithecellobium spp.* is found on BHSP. No *C. bonduc* has been found on the islands of KWNWR with *C. t. bethunebakeri*. Historical data show that larvae were able to feed on both host plants as well as *Cardiospermum spp.* (Sapindaceae). An ancestral polymorphism explains how larvae of the same taxon can be feeding on very different plant families. Adaptation to specific hosts can lead to changes in allelic frequency (Sunnucks et al. 1997) and may lead to speciation (Templeton 1981) and, taken together, these results impress the need for further study.

This study further clarifies the fact that the underlying genetic diversity of *C. t. bethunebakeri* is not due to an accelerated rate of mutation of microsatellite markers. The mutation rate for this taxon at these microsatellite loci is 1.98×10^{-3}, which falls well within the range of published mutation rates across taxa and especially when compared to related invertebrate taxa; an ant at 1.8×10^{-3} (Crozier et al. 1999) and a freshwater snail at 1.1×10^{-3} (Gow et al. 2005). The extensive survey of wheat microsatellite marker mutations over 22 generations showed similar rates ($0 - 4.97 \times 10^{-3}$ per generation) and that the genetic diversity in the initial parental populations was conserved over time. Taken together, these results show that microsatellite mutations, i.e. the emergence of new diversity, balance the loss by selection and drift (Raquin et al. 2008). Loss of diversity by drift is of special concern in small populations, like those of *C. t. bethunebakeri*.

The BHSP population has persisted since at least 1999, despite subjugation to both major hurricane events and severe droughts between 2002-present. This taxon has essentially persisted in the face of natural disaster yet has been unable to cope with the human-induced disturbance

and historical destruction of habitat that have affected its former habitat in south Florida. The data presented here speak to the fact that the habitat patches and metapopulation structure allow for significant gene flow, maintenance of genetic diversity, and reduced levels of inbreeding; all of which are essential to the conservation of this taxon. This situation mirrors that of the Glanville fritillary *Melitaea cinxia*, which went extinct on the Finnish mainland in the 1970s and now occurs on neighboring islands in a fragmented, metapopulation structure. Extensive study of this system has shown how increased habitat fragmentation furthers colony extinction and how a balance between local extinctions and recolonizations are essential for taxon survival (Hanski et al. 1995; Hanski & Ovaskainen 2003). Additional studies of neotropical trees further highlight the importance of gene flow in maintaining genetic diversity and supporting populations over time (Lowe et al. 2005). Host plant quality may vary over time and space, but the presence of a variety of habitat patches ensures that some suitable host is always present for *C. t. bethunebakeri* butterflies and larvae. Fine-scale habitat changes may influence the development of individuals, but the overall health of the taxon can be ensured by retaining a variety of such habitats.

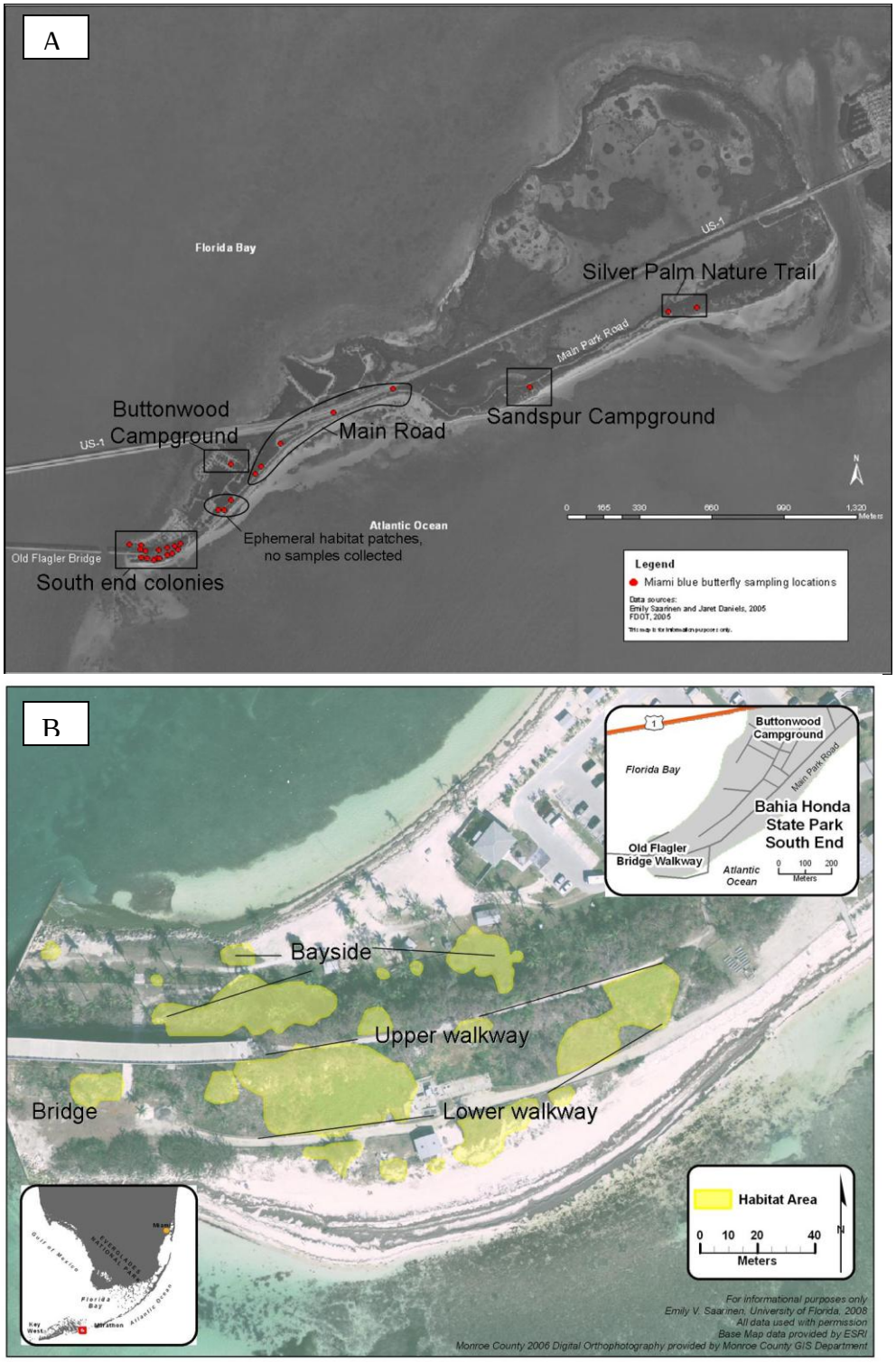


Figure 4-1. Schematic diagram of Bahia Honda State Park, Florida *C. t. bethunebakeri* sampling locations. A, overview; B, detail of South end colonies.

Figure 4-2. Removal of a wing fragment from an adult *C. t. bethunebakeri* butterfly for use in genetic analyses.

Table 4-1. Sampling regime for molecular studies of C. t. bethunebakeri on Bahia Honda State Park, Florida 2005-2006.

Date	*N*	*N* (pooled samples)
September 2005	24	24
April 2006	4	
May 2006	17	21
June 2006	39	
July 2006	9	48
September 2006	8	
October 2006	13	21
Total 2005	24	
Total 2006	90	
Total	114	

Table 4-2. Genotyping error rates for each microsatellite locus of the *C. t. bethunebakeri*. *N*, number of DNA samples with second round of PCR and genotyping; % matching, percentage of second genotyping results matching original score. All samples are from Bahia Honda State Park, Florida.

Locus	*N*	Correct allele matches	Incorrect allele matches	% matching	% failure due to allelic dropout
CthB103	3	6	0	100.00%	NA
CthB106	12	14	10	58.33%	80%
CthB115	13	24	2	92.31%	50%
CthB117	9	14	4	77.78%	75%
CthB119	11	14	8	63.64%	100%
CthC12	12	24	0	100.00%	NA
CthC116	10	16	4	80.00%	50%
CthC124	5	10	0	100.00%	NA
CthD7	23	46	0	100.00%	NA
Total	127	168	28	85.71%	78.57%

Table 4-3. Summary statistics of allelic patterns for *C. t. bethunebakeri* in Bahia Honda State Park (BHSP), Florida. Values are averaged across the 12 loci; *N*, sample size; n_e, mean number of effective alleles; H_O, mean observed heterozygosity; H_E, mean expected heterozygosity. Data from Key West National Wildlife Refuge (KWNWR) are given last.

Population (*N*)	Total alleles	Private alleles	n_e	Mean H_O	Mean H_E
September 2005 (24)	52	5	2.064	0.365±0.067	0.458±0.057
April+May 2006 (21)	50	2	2.199	0.421±0.075	0.484±0.060
June 2006 (39)	64	10	2.134	0.425±0.073	0.463±0.060
July 2006 (9)	40	1	2.077	0.350±0.068	0.445±0.059
June+July 2006 (48)	67	13	2.125	0.413±0.064	0.463±0.060
September 2006 (8)	37	3	2.388	0.268±0.084	0.425±0.074
October 2006 (13)	42	7	2.272	0.420±0.074	0.513±0.054
September+October 2006 (21)	53	10	2.308	0.362±0.063	0.508±0.053
Total 2005 (24)	52	5	2.064	0.365±0.067	0.458±0.057
Total 2006 (90)	84	34	2.246	0.403±0.060	0.494±0.057
Total BHSP (114)	92	56	2.233	0.395±0.060	0.493±0.057
KWNWR (27)	58	22	3.010	0.510±0.058	0.601±0.051

Table 4-4. Hardy-Weinberg equilibrium (HWE) probabilities for *C. t. bethunebakeri* in BHSP and KWNWR populations by locus. *N*, sample size in parentheses. Sequential Bonferroni corrections have been done for each population to correct Type 1 error. Locus CthC124 is monomorphic (M) in three populations. *P*-values in italics are in HWE. All data are discrete populations and no data are pooled between months.

Locus	BHSP September 2005 (24)	BHSP May 2006 (21)	BHSP June 2006 (39)	BHSP October 2006 (13)	KWNWR 2008 (27)
CthB11	*0.029*	*0.201*	*0.028*	*0.516*	*0.549*
CthB101	*0.280*	0.005	0.000	*0.071*	*0.174*
CthB103	*0.147*	*0.058*	*0.403*	*0.600*	*0.010*
CthB106	0.002	0.004	0.003	*0.036*	*0.080*
CthB115	*0.045*	*0.186*	0.001	0.000	0.000
CthB117	0.000	*0.043*	*0.126*	0.001	*0.010*
CthB119	0.000	0.004	*0.513*	0.004	0.002
CthC12	0.001	0.000	0.000	0.001	*0.316*
CthC116	*0.599*	*1.000*	*0.025*	*0.518*	*0.428*
CthC124	M	M	*0.027*	M	*0.136*
CthC127	*0.021*	*0.420*	*0.561*	*0.393*	*0.347*
CthD7	*0.184*	*0.077*	0.000	*0.365*	*0.897*

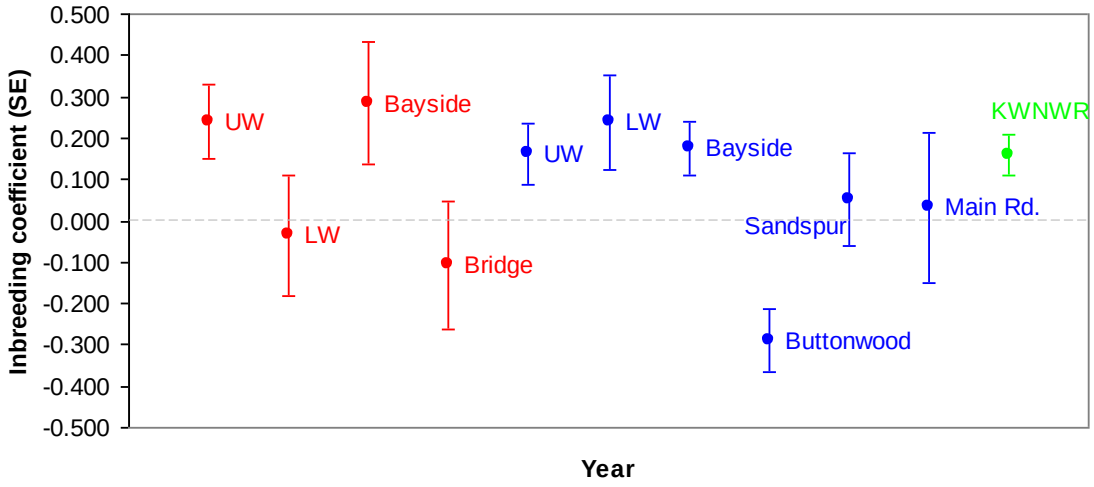


Figure 4-3. Inbreeding coefficients (and standard error bars) for each metapopulation segment of C. t. bethunebakeri on Bahia Honda State Park over time (2005-2006). Red, 2005; Blue, 2006; Green, 2008; UW, upper walkway; LW, lower walkway; Bayside, Florida Bay side of BHSP; Bridge, base of Old Flagler Railroad; Buttonwood Campground; Sandspur Campground; Main Road of BHSP. Data from the Key West National Wildlife Refuge (KWNWR) from the Marquesas Islands are also shown.

Table 4-5. Wilcoxon signed rank tests for excess (top) and deficiency (bottom) of heterozygosity for discrete breeding populations of wild and captive-raised laboratory populations of *C. t. bethunebakeri*. BHSP, Bahia Honda State Park; CC, captive colony; KWNWR, Key West National Wildlife Refuge. Significant values ($P < 0.05$) are in italics; IAM, infinite allele model; TPM, tow-phase model; SMM, stepwise mutation model.

Population	Sample size	IAM	TPM	SMM
BHSP-9-2005	24	0.618/0.416	0.913/0.103	0.992/*0.011*
BHSP-5-2006	17	0.416/0.617	0.817/0.207	0.989/*0.027*
BHSP-6-2006	39	0.849/0.170	0.954/0.055	0.993/*0.017*
CC-7-2006	40	0.661/0.367	0.979/*0.026*	0.999/*0.001*
CC-10-2006	10	*0.003*/0.998	0.103/0.913	0.289/0.740
CC-11-2006	12	0.150/0.875	0.327/0.715	0.545/0.500
KWNWR-2-2008	27	*0.003*/0.997	0.088/0.924	0.689/0.339

Table 4-6. Effective population size (N_E) estimates of the BHSP population of *C. t. bethunebakeri*; May 2006-October 2006. Results were generated by comparing allelic frequencies in consecutive generations in a moments based temporal model.

Generation	# generations	N_E	95% CI
May - June	2	26.6	10.0 – 534.6
June - July	2	45.5	9.2 - ∞
May - July	3	25.1	8.2 - ∞
September - October	2	5.4	2.3 – 17.2
May – October*	6	26.8	12.5 – 68.8

* There is no data for August 2006.

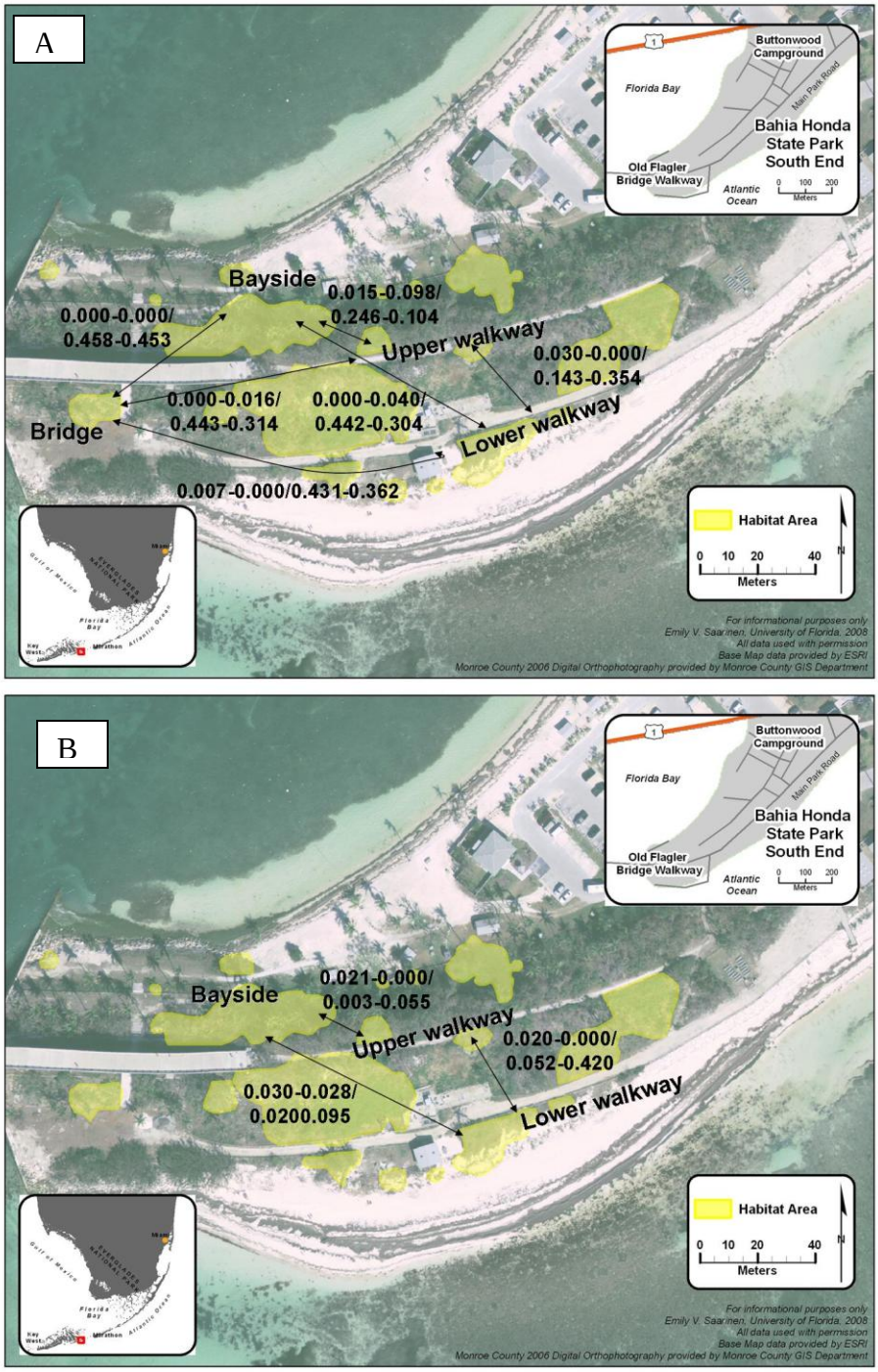


Figure 4-4. Pairwise F_{ST}-R_{ST} and corresponding *P*-values (after diagonal) for *C. t. bethunebakeri* on BHSP. A, 2005; B, 2006. *P*-value significance calculated from 9,999 permutations of 12 microsatellite loci. Low sample size prevented Bridge 2006 values from being calculated.

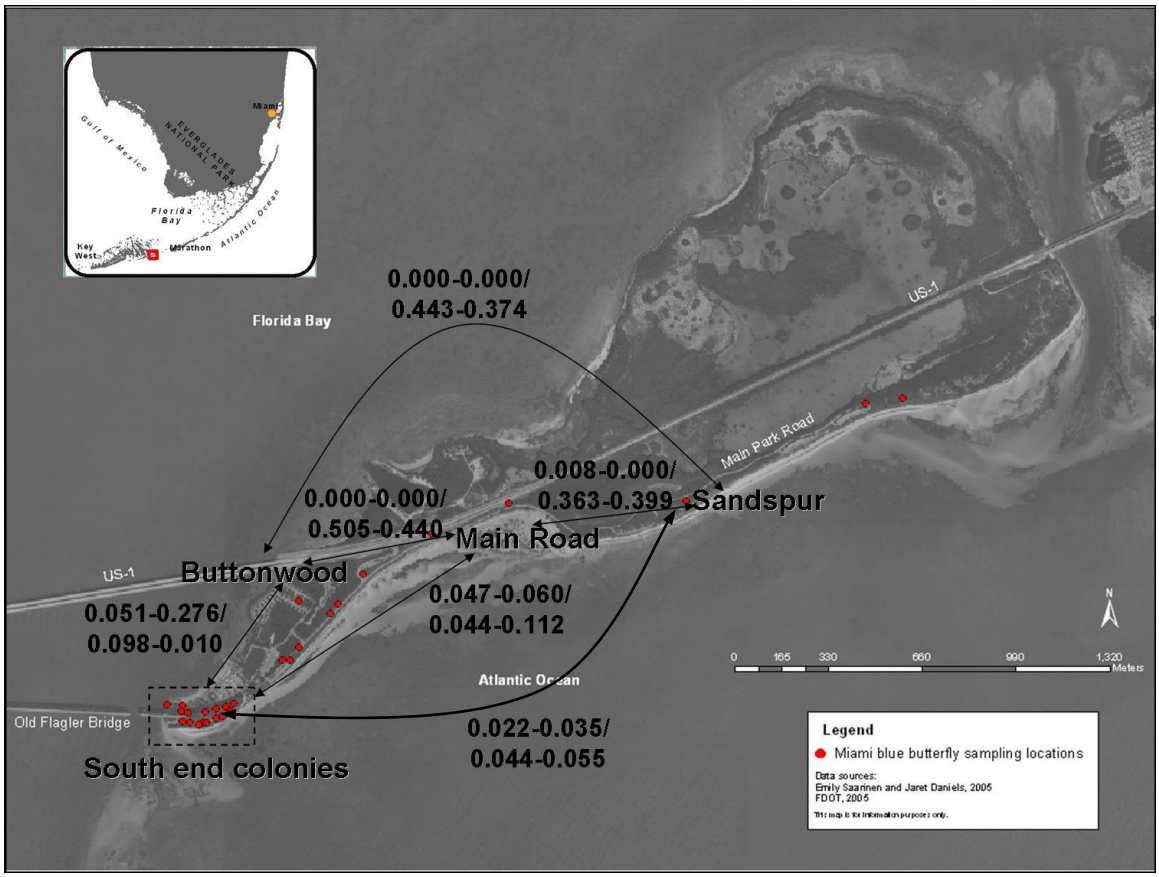


Figure 4-5. Pairwise F_{ST}-R_{ST} and corresponding *P*-values (after diagonal) for *C. t. bethunebakeri* on BHSP in 2006. *P*-value significance calculated from 9,999 permutations of 12 microsatellite loci.

Table 4-7. Results from Structure 2.0 for *K* values 1-5, data are from all BHSP subpopulations (*N* = 114) and are not pre-assigned to populations for analysis. The highest Ln *P*(*X*/*K*) in 5 runs is shown with mean inferred assignment to clusters.

K	Ln *P*(*X*/*K*)	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5
1	-2688.1					
2	-2570.4	0.462	0.538			
3	-2624.4	0.298	0.336	0.366		
4	-2651.4	0.238	0.243	0.265	0.254	
5	-2780.8	0.197	0.188	0.210	0.206	0.199

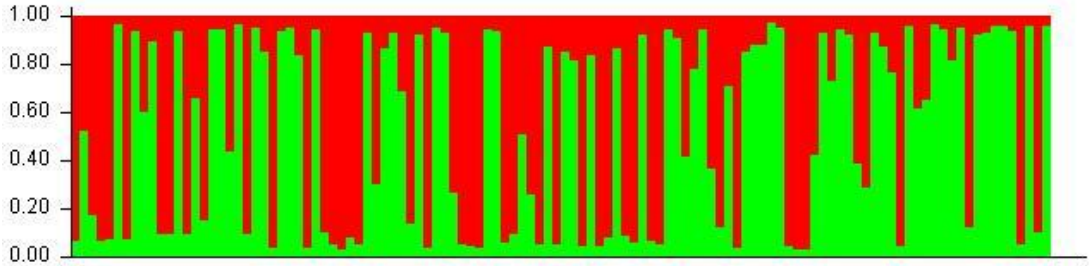


Figure 4-6. Structure plot of all BHSP data (N =114) when K = 2, data are from all BHSP subpopulations (N = 114) and are not pre-assigned to populations for analysis. Each vertical line represents one individual butterfly; red and green represent two different source populations. Data are shown chronologically, with BHSP-9-2005 on the left and ending with BHSP-10-2006 on the right.

Table 4-8. Results from Structure 2.0 for K values 1-5, data are from the BHSP-9-2005 (N = 24) and KWNWR-2-2008 (N = 27) populations. The highest Ln $P(X/K)$ in 5 runs is shown with mean inferred assignment to clusters. Prior pop., samples divided into two source populations based on location; No prior pop., samples not pre-assigned to a population for analysis.

K	Prior pop. Ln $P(X/K)$	No prior pop. Ln $P(X/K)$	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5
1	-1327.0	-1327.1					
2	-1187.7	-1189.1	0.491	0.509			
3	-1243.6	-1172.4	0.385	0.280	0.335		
4	-1225.1	-1155.4	0.263	0.235	0.240	0.262	
5	-1261.6	-1135.6	0.210	0.200	0.191	0.226	0.173

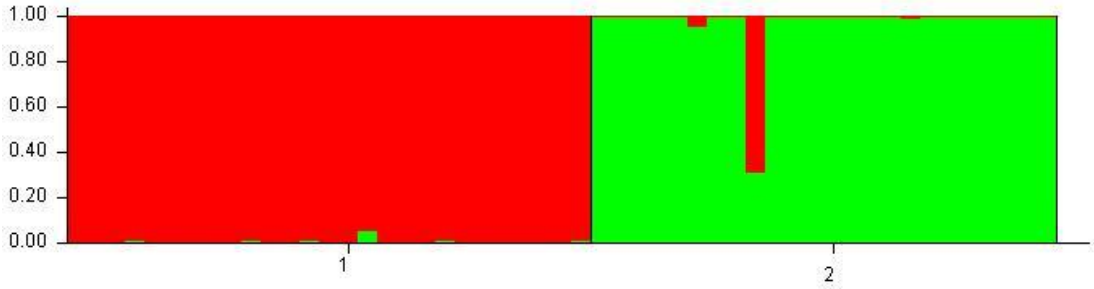


Figure 4-7. Structure plot of KWNWR-2-2008 (N = 27) and BHSP-9-2005 data (N = 24) when K = 2 and prior population assignment is incorporated into the model. Each vertical line represents one individual butterfly; red and green represent two different source populations; 1, KWNWR; 2, BHSP.

CHAPTER 5
GENETICS OF THE CAPTIVE COLONY

Introduction

Numerous animal and plant species are endangered in the wild and now rely upon captive breeding for survival or as insurance against extinction (Soulé et al. 1986; Frankham 2008). The maintenance of genetic diversity in a captive colony should be a priority when individuals produced therein will be released to found wild populations. A captive colony must therefore actively capture and reflect the genetic diversity present in remaining wild populations. Genetic monitoring of captive colonies can be used to detect changes in genetic diversity over generations in captivity and to measure potential effects of inbreeding or drift (Lacy 1993). It is additionally important to genetically monitor individuals for signs of genetic adaptation to captivity as this is expected to have negative effects on reintroduction success (Frankham 2008).

The Miami blue butterfly, *Cyclargus thomasi bethunebakeri*, was once a common taxon throughout southern Florida but is currently only found on two island regions (Bahia Honda State Park and Key West National Wildlife Refuge) in the extreme southern portion of the state (Klots 1964; Lenczewski 1980; Minno & Emmel 1993; Ruffin & Glassberg 2000; Calhoun et al. 2002; Cannon 2007). The documented decline may have begun gradually, but it has become dramatic within recent years. *Cyclargus thomasi bethunebakeri* is currently one of the most endangered taxa in the United States due to its low population numbers, presence at a fraction of its original geographic range, and susceptibility to impact from disturbances such as hurricanes and tropical storms. Through public petition, this taxon was given emergency status as a state-endangered species in Florida in November 2002 by the Florida Fish and Wildlife Conservation Commission (FWC) and an emergency management plan was later drafted. *Cyclargus thomasi bethunebakeri* is currently a candidate to be listed as a federally-endangered species.

The Florida Fish and Wildlife Conservation Commission (FWC), Department of Environmental Protection (DEP), U.S. Fish and Wildlife Service (USFWS), and the University of Florida (UF) jointly agreed to establish a captive propagation program due to the extremely low estimated population size of less than 50 adult individuals remaining in Bahia Honda State Park (BHSP). The FWC authorized the collection and transport of 100 *C. t. bethunebakeri* eggs to the University of Florida in Gainesville for the purpose of establishing a captive breeding population. The purposes of this colony are to conserve the remaining genetic diversity of the taxon, safeguard the existence of this taxon from natural and manmade factors that may threaten its existence, and to produce a significant number of individuals for reintroduction into the wild.

Given the negative genetic effects that occur in captive breeding (inbreeding depression, adaptation to captivity, loss of diversity), it is important to assess the genetic constitution of a captive colony (Frankham 2008). It is not only important that the captive colony reflect the genetic diversity within the wild colony on Bahia Honda State Park, but that it continues to mirror this wild population over time and that adaptation to captivity is minimized. In this study I assess how genetic diversity is lost in captivity through comparison of captive generations over time as well as through direct comparison with the founding population on Bahia Honda. I also evaluate the captive colony for novel mutations as well as changes in effective population size.

Methods

Basis of Captive Colony

A total of 100 eggs were collected from ten randomly captured female *C. t. bethunebakeri* originating from several sites within BHSP over a four month period from February 2003 to May 2003. The resulting 100 eggs were used as the foundation for building the captive colony at the University of Florida. In order to avoid marked inbreeding depression, captive breeding was based on a founder number of at least ten unrelated pairs (Saccheri et al. 1996). To maintain

genetic diversity over time and to compensate for lower founder numbers, captive populations were allowed to increase rapidly after establishment and future population bottlenecks were avoided as possible (Saccheri et al. 1996; van Oosterhout et al. 2000; Saccheri et al. 2001).

A significant decline in captive colony numbers was experienced between November 2005 and February 2006 (Table 5-1). Subsequent analysis by University of Florida virologists Dr. James Maruniak and Dr. Aléjandra Maruniak confirmed the presence of a baculovirus in the captive colony. The presence of baculovirus infections can be common in lepidopteran populations, and can be particularly devastating and difficult to control in captive or laboratory situations (Crone et al. 2007). Due to the resulting significant decline in larval numbers, the FWC and University of Florida jointly agreed to terminate the initial captive colony after some 30 generations and start over with new founder stock from BHSP.

Collection of new genetic material from BHSP was initiated in early April 2006 and continued through June 2006. A total of 80 eggs were collected as initial founder stock from multiple locations within BHSP (Table 5-2).

Incorporation of New Genetic Material

The captive colony received the addition of new genetic material when the removal of material from BHSP was possible without further depressing the population. A total of 100 eggs (25 per quarter) were collected and transported to the University of Florida each year (FWC permit amendment no. WX02525a) when they were available from BHSP. Due to large captive colony numbers typical of many insect populations, traditional studbook methods widely used for other breeding programs (primarily mammalian) were unrealistic in this situation due to funding, time and manpower restrictions. Alternative methods, similar to those in fish captive breeding programs, were therefore used to manage and conserve available genetic diversity in the butterfly captive colony (Fiumera et al. 1999). These methods included the maintenance of

detailed records on the origin and mating of founder lines. Males were allowed to mate only a single time and sibling mating was restricted. Various life history and behavioral data, particularly as they related to overall organism fitness, were recorded when appropriate. All changes or deviations from the norm were noted and used as a yardstick to better evaluate overall colony health. Similarly, efforts were made to maintain captive colony numbers between 150-400 organisms and to reduce rapid fluctuations in overall colony numbers.

Sampling of Captive Colony

A sample of butterflies that died of natural causes in the captive colony were saved and kept in a -20℃ freezer beginning in 2004. These butterflies were used to assess the genetic diversity of the captive colony over time. Butterflies from several generations were stored in groups in 2004 and thus knowledge of discrete generations during these years was not available. Data from the 2004 captive colony therefore represents 1-12 generations (Table 5-1). Butterflies in subsequent years were kept separate to allow for analyses of discrete generations. DNA was extracted from the thoraces of butterflies and twelve polymorphic microsatellite loci were PCR-amplified according to methods in Saarinen et al. (2009). Data from captive colony butterflies were compared with BHSP data from 2005-2006.

Statistical Analyses

Microsatellite data were organized in Microsoft Excel and formatted using Convert (Glaubitz 2004) to be used in other software packages. GenAlEx 6.1 (Peakall & Smouse 2006) was used to calculate the observed number of alleles, effective number of alleles per locus, lists of private alleles per locus, and the observed and expected heterozygosities. Statistical difference between means of genetic diversity values are assessed by paired *t*-tests as recommended by Nei (1987). I used FSTAT version 2.9.3.2 to test for allelic richness (A_R), a measure of the number of alleles independent of sample size (Goudet 2001). The independence

from sample size allows for the comparison between generations or populations of varying sizes. GenAlEx was used to calculate Nei's genetic distance measurements as per Nei (1972; 1978). Nei (1972) details D_S, a traditional genetic distance measure with relatively low variance that measures the accumulated allele differences per locus. Nei (1978) describes the unbiased estimator of genetic distance, and this statistic has been recommended to correct for differences in low sample size (Paetkau et al. 1997) although Nei himself (1978) cautions that when genetic distances are minimal, more individuals need to be sampled. Nei (1978) further notes that the unbiased genetic distance estimator works well with most sample sizes as long as more than 30 loci are used. Hedrick (2005) further cautions against use of the unbiased estimator of genetic distance as it may give poor results when homozygosity and sample size are small. As there are 12 polymorphic loci used in the study of *C. t. bethunebakeri*, I present both the traditional and unbiased measures of Nei's genetic distance.

The program Bottleneck (Cornuet & Luikart 1996) was used to look for genetic signatures typical of a population that has undergone a bottleneck. The Stepwise Mutation Model (SMM), Infinite Alleles Model (IAM), and Two-Phase Model (TPM) of microsatellite evolution were calculated, allowing 30% variance for TPM with a 70% proportion of the SMM and 1000 iterations of all models. I tested a random sample of adults from different generations of the captive colony that approximately met the sample size criterion of 15-40 individuals (Cornuet & Luikart 1996). To ensure that the program worked with my data, I also tested a known population bottleneck using data from the October 2006 captive colony. This iteration of the captive colony had been allowed to breed without the introduction of new genetic material for four generations, thus representing a known population bottleneck (Table 5-2). I used the program NeEstimator (Peel et al. 2004) to estimate the effective population size (N_E) of the

captive colony of butterflies. This program uses a moments-based temporal model (Waples 1989) to estimate the effective population size over time. For these analyses, consecutive generations were required to estimate effective population sizes. As I did not have data from the captive colony for August or September 2006, these are not factored in the analyses. Estimates of the effective size of the captive colony were compared to estimates of the BHSP colony.

Results

The captive propagation program has been extremely productive since its inception in February 2003 yielding almost 25,000 viable *C. t. bethunebakeri* adults (Table 5-1 and 5-2). It has served as the basis for the successful production of organisms for reintroduction as well as ecological, behavioral, and non-target pesticide research.

Genetic Changes in the Captive Colony over Time

Observed heterozygosity remained relatively constant across different generations of the captive colony (Table 5-3). However, when looking at successive generations of the 2006 captive colony, observed heterozygosity did fall significantly from June (H_O = 0.410) to November (H_O = 0.297) (P = 0.042, df = 11). One of the last generations of the initial captive colony (November 2005) had an observed heterozygosity of 0.373 and one year later in November 2006 observed heterozygosity had decreased to 0.297, although not significantly (P = 0.105, df = 11). When genetic data were pooled and examined across years rather than generations, the observed heterozygosity remained relatively constant and no statistical significances were found between years (H_O = 0.332 in 2004, H_O = 0.373 in 2005, and H_O = 0.377 in 2006; P = 0.861, $F_{2,33}$ = 0.150). A general trend of loss of allelic richness was observed over time (Figure 5-1). Allelic richness in the November 2005 captive colony was 3.673 and it was 2.360 one year later in 2006 (P = 0.0004, df = 11). Other measures, such as the number of

effective alleles (n_e) was relatively constant over both generations and years (Table 5-3), however low n_e values were again seen in the November 2006 captive colony.

The effective population size (N_E) of the first iteration of the captive colony could not be calculated because materials were not saved from multiple discrete generations. In the 2006 captive colony, there was a low effective population size of each generation of the captive colony of less than 15 individuals (1.7-47.5 individuals, 95% CI). The effective population size decreased from the summer to the fall of 2006 as there was no new genetic material incorporated into the colony during this time (Table 5-4).

Changes in the captive colony were also measured using the accumulated allele differences per locus to yield the genetic distance between generations. Measures of genetic distance are presented as pairwise population matrices of Nei's genetic distances (traditional, Table 5-5 and unbiased, Table 5-6). These figures can be divided into three data quadrants. The lower right quadrant shows the genetic distances between discrete generations of the captive colony. Each column of data in a quadrant shows that genetic distance increases with time in both the traditional and unbiased measures. All unbiased measures of distance are lower than traditional values, although they show the same trend of increasing with comparisons of more temporally-separate generations. The upper left quadrant shows changes in genetic distance in generations of butterflies from BHSP and is discussed in the following section. Genetic distance measurements from population matrices are plotted in Figures 5-2 and 5-3.

Comparisons with Bahia Honda State Park

The BHSP population and captive colony have held a similar number of alleles (N_A) and similar number of effective alleles (n_e) over time. Both BHSP and the captive colony held ~2 effective alleles from 2004-2006 (Table 5-3). Measures of observed heterozygosity were also

similar, but no real temporal trends were evident (Figure 5-4). Allelic richness was lost over time in the captive colony but remained stable in BHSP (Figure 5-1).

Changes in genetic distance between generations of the captive colony and BHSP population were evident (Tables 5-5 and 5-6, lower left quadrants). Again, trends were similar in both tables although unbiased estimates of genetic distance were lower than traditional estimates. One generation was required for genetic material collected from BHSP to become incorporated and detectable in the captive colony. For this reason, a series of pairwise comparisons between months of generations is shown for BHSP and the captive colony. Generational data was also pooled for comparison of distance between the wild and the captive colony over years.

Comparison of discrete generations showed an increased genetic distance between BHSP and captive colonies over time (Table 5-5 and 5-6). It is important to remember that a new captive colony was initiated in 2006. Comparison between the first captive colony and the BHSP showed traditional $D_S = 0.138$ and unbiased $D_S = 0.113$ (Tables 5-5 and 5-6). When generations (months) were compared in the second iteration of the captive colony (e.g., BHSP June 2006 vs. CC June 2006, BHSP July 2006 vs. CC July 2006, and BHSP October 2006 vs. CC October 2006), genetic distance between the BHSP and captive colonies increased over time from June to September 2006. This increase in genetic distance over time between wild and captive populations was also evident when a lag time (allowing for incorporation of new material) was factored into the analysis (Table 5-5 and 5-6). A decrease in genetic distance between the October 2006 BHSP population and the November 2006 captive colony was also observed.

Genetic distance values between generations were high (mean traditional $D_S = 0.114$, mean unbiased $D_S = 0.083$) when considered relative to the genetic distance calculated between years.

When all BHSP 2006 data were pooled and compared against all CC 2006 data, distance values were 0.036 and 0.030, for traditional and unbiased estimations of genetic distance, respectively. When data were pooled over the two years 2005-2006, values decreased to 0.027 (D_S) and 0.022 (unbiased D_S).

Genetic distance values over time within the BHSP population did not change, but the captive colony showed significant changes (Figures 5-2 and 5-3). Not only were changes in genetic distance higher in the captive colony, but they also increased over time. Again, changes in observed heterozygosity over time in wild and captive colony samples showed no real trends (Figure 5-4).

Private Alleles

All private alleles conformed to base pair size expectations, but there were unique alleles only found in the BHSP population as well as private alleles only found in the captive colony (Appendix D). When considering the entire pool of alleles sampled from BHSP in 2005-2006 (N = 114) vs. the alleles sampled from the captive colony during these same years (N = 101), there were 29 alleles found only in BHSP and 17 alleles found only in the captive colony. Of these 29 alleles, 25 were found at a low frequency of 0.004-0.009 in BHSP. The remaining four alleles were found at the intermediate frequencies of 0.013-0.022. In the captive colony, there were 17 alleles not found in BHSP. Of these alleles, only 6 were found at a low frequency (<0.009). Nine alleles were found at an intermediate to high frequency of 0.020-0.067. In summary, there were 29 alleles not represented in the captive colony from BHSP and there were 17 novel mutations in the captive colony not sampled or not present in BHSP. Of the private alleles in BHSP, 86% were found at a low frequency, versus 35% of private alleles in the captive colony found at low frequency.

Bottlenecks

Different mutation models had an effect on the detection of heterozygote excess and deficiency (Table 5-7). The IAM was the only model able to detect a population bottleneck (denoted by heterozygote excess) in the October 2006 captive colony. The TPM and SMM failed to detect any bottlenecks. The program Bottleneck also detected a number of heterozygote deficiencies (Table 5-7). The TPM detected a heterozygote deficiency in the July 2006 captive colony, the SMM detected heterozygote deficiencies in the June and July 2006 captive colonies, and the IAM detected no heterozygote deficiencies.

Discussion

The captive colony has been successful at generating individuals for reintroduction to the wild and in safeguarding the remaining wild populations. The captive colony has had the additional benefit of elucidating life history characteristics for immature and adult stages, including the identification of a baculovirus that was capable of decimating the captive colony and could potentially destroy wild colonies as well. Information gathered from captive colony management is not always directly applicable to natural populations, but it may highlight various stochastic factors that are of importance in the wild as well, such as the devastating effects of a pathogen.

The genetic measures used in this study (N_A, n_e, H_O, A_R, D_S, and N_E) were useful in evaluating the negative consequences of captive breeding. Together these methods are useful in elucidating the similarities and differences in the captive colony and BHSP over time as well as genetic diversity lost in the captive colony. Individually, these measures can highlight novel mutations that have arisen in captivity and provide other estimates of genetic makeup such as effective population size. A concern in captive colony management is whether genetic diversity is lost over time (as a result of inbreeding or genetic drift) (Briscoe et al. 1992; Woodworth et al.

2002; Frankham 2008). The number of alleles (N_A) is biased by sample size, but when considering successive generations of similar sample size, a declining trend in the number of alleles in the captive colony is evident over time, and the number of effective alleles (n_e) shows a similar trend. Values of observed heterozygosity fell significantly from November 2005 to November 2006 as did measures of allelic richness. Taken together, these data confirm that genetic diversity has been lost over time (between generations) of the captive colony. However, when years are compared instead of generations, all diversity measures are more stable. This is due to the influx of new genetic material during some generations and a complete lack of immigration during others. More effort was made in incorporating genetic material from BHSP into the captive colony on a regular basis, but drought conditions in the summer of 2006 often prevented this. New genetic material was not added to the captive colony from July 2006-May 2007 as BHSP suffered from drought conditions and low population estimates (J.C. Daniels, unpublished data). The captive colony was not infused with new genetic material for approximately one year and evidence of genetic divergence from BHSP frequencies sampled was observable within three months. Decreases in genetic diversity measures were seen during these times and fewer individuals were produced in the captive colony. Allelic richness also decreased during this time. This denotes the importance of bringing new material into a captive colony on a generational or bi-generational basis both to maintain genetic diversity and to prevent adaptation to captivity (Frankham & Loebel 1992).

Another concern in captive colony management is how different (in terms of genetic distance, allelic frequencies, and novel alleles) the captive population becomes when compared to a wild population. Captive colony census numbers decreased in winter months and increased as conditions improved, much as they did in BHSP (J.C. Daniels, unpublished data). There were

high levels of gene flow and, coupled with the effects of natural selection, the BHSP population remained "genetically consistent" over time (E.V. Saarinen, unpublished data). The captive colony is not subjected to natural selection, allowing genetic drift to have a greater impact on allelic frequencies and as such, result in greater genetic distances both between successive generations and when compared with the BHSP population. However, as the markers used in this study are neutral and presumably not linked to fitness measures, they would not be under selection in either the wild or the captive population and the differences in frequency are due to random chance coupled with effective population size differences in each population.

Private Alleles

Differences between the BHSP population and the captive colony were due to differences in allelic frequencies as well as the presence of private alleles in both groups. These alleles were found only in single populations and usually occurred at low frequency. Private alleles found only in BHSP represent genetic diversity that has not been sampled and incorporated into the captive colony. Most private alleles in BHSP were present at a low frequency, indicating that they may be new mutations. These potentially novel mutations will either be lost (by drift) or retained in the population. It will be important to monitor these alleles over time and to incorporate them as necessary into the captive colony. Care should be taken to see where individuals with these private alleles are found on BHSP so that individuals from these habitat patches may be incorporated into the captive colony. It is difficult to assess whether the novel alleles sampled from the captive colony represent real adaptation to captivity or simply neutral variation as they are from neutral microsatellite markers. These alleles underscore the importance of drift in captivity as they are the products of new mutation events that have drifted to a higher frequency in the absence of selection. In BHSP, alleles are lost during the natural selection process. In the captive colonies, non-lethal molecular diversity is preserved. Private

alleles in the captive colonies may not be selectively advantageous in a natural setting. In fact, these alleles may be neutral or deleterious (or linked to a deleterious trait), underscoring the importance of keeping captive colonies genetically similar to wild populations in order to produce the best fit individuals for reintroduction.

More sampling has occurred in the captive colonies than from BHSP. A greater sampling coverage will return more alleles if all of the diversity was not captured by smaller sampling endeavors. The private alleles from the captive colonies may be present in BHSP at low levels but may not have been sampled.

Bottlenecks

New genetic material was last incorporated into the captive colony in June 2006, so it is understandable that a bottleneck signature would appear four months later. It is unclear why there was no bottleneck detected in the November 2006 captive colony. The IAM was the best model for detecting a population bottleneck utilizing microsatellite data, while the SMM performed the best for detecting heterozygote deficiencies. Heterozygote deficiencies were detected the generation after new material was incorporated into the captive colony from the wild, but disappeared in subsequent generations. This confirms the conclusion made by Harper et al. (2003) that both the TPM and SMM perform poorly at detecting past bottlenecks and I emphasize the importance of utilizing several mutational models.

Effective Population Size

The effective population size of the captive colony is extremely low and represents only a fraction of the census size of the captive colony. The effective population size of the captive colony is also lower than the BHSP effective population size, a situation similar to other captive colonies (Bailey et al. 2007). Such figures are important as they show the importance of genetic diversity, not just maintaining a large captive population (Briscoe et al. 1992).

Implications for Conservation

The markers used in this study were presumably neutral and it is therefore not possible to differentiate whether allelic changes are due to drift or adaptation to captivity in the BHSP and captive colony populations. The fact that there are 17 alleles only found in the captive colony and that these alleles are found at moderate frequencies, speaks to their presence as an adaptation to captivity. A mutation in the wild has the chance of drifting to a higher frequency if there are no selection pressures against it, and this may be the case in captivity. These novel mutations may be able to drift to a higher frequency in captivity but may be deleterious in the wild. The accumulation of novel mutations such as these may eventually lead to "mutational meltdown" and fitness measures should be monitored closely in the captive colony (Woodworth et al. 2002).

This study shows how, at neutral markers, a captive colony will diverge from a wild population over time. This has many conservation implications if individuals produced in the captive colony are to be used for reintroduction purposes. Multiple procedures should be followed in order to reduce genetic adaptation to captivity. The first method is to introduce immigrants from the wild into the captive colony on a regular basis, once per generation if possible (Woodworth et al. 2002; Frankham et al. 2003). This is not possible for many endangered species programs as there are no remaining wild populations or to remove an individual would be unwise. The *C. t. bethunebakeri* program maximizes on the ability to incorporate wild stock into the captive program on a regular basis, and this study highlights the negative repercussions of not doing so. Next, individuals should be raised in environments as similar to wild conditions as possible. Again, this is not possible due to funding and facility restrictions for most taxa. Individuals from the captive colony of *C. t. bethunebakeri* are allowed to mate in outdoor flight cages and are subjected to environmental changes as adults but other than that, conditions are kept very benign and do not afford any type of selection. Two other

procedures that have been recommended by multiple authors (Margan et al. 1998; Fiumera et al. 1999; Woodworth et al. 2002; Frankham et al. 2008; Fraser 2008) have not yet been instituted in this captive management program. These recommendations are to 1) equalize family size and 2) fragment captive populations. Frankham et al. (2000) show that the equalization of family sizes effectively slows genetic adaptation to captivity in *Drosophila* and is a technique that could be easily done with butterflies. This procedure could be carried out in conjunction with the next objective of fragmenting the population in multiple colonies. Family sizes could be equalized in different populations, thus removing the need to cull larvae from the program. Fragmenting the existing colony would help to safeguard it from future baculovirus or other pathogen outbreaks as well as to slow genetic deterioration (Margan et al. 1998). Genetic adaptation to captivity is more significant in large populations than small populations (Woodworth et al. 2002), but small populations show greater inbreeding depression. This balance may be achieved by subdividing a captive colony into several small-medium sized captive populations. Smaller captive colonies are also easier for technicians to manage and smaller populations show less response to selective pressures (Woodworth et al. 2002).

Lastly, because both wild and captive populations have private alleles, it is theoretically possible that they could enhance each others' fitness through the introduction of novel genetic diversity (Bailey et al. 2007, but see Ford 2002). However local adaptations, both in captivity and in the wild, likely outweigh any benefit from the introduction of novel alleles as well as the potential breakdown by outbreeding depression. Theodorou and Couvet (2004) have shown that captive-bred individuals may have a positive effect on dwindling wild populations if a strict model is followed, including few captive-breeders released per generation and adherence to captive-breeding protocols. As such, captive-bred individuals should only be used to found new

colonies and should spend as few generations in captivity as possible. Captive-breeding programs require extensive amounts of time and energy (Snyder et al. 1996) and if their ultimate goals are to produce reproductively-fit offspring, genetic variability must be maximized and genetic adaptation to captivity minimized.

Table 5-1. Summary of *C. t. bethunebakeri* initial captive colony numbers from February 2003 to December 2005. *N*, number of Bahia Honda State Park (BHSP) collected females refers from which eggs originated and were incorporated to the captive colony; *N* eggs collected from BHSP were directly collected from BHSP and added to the captive colony.

Captive generation	Month/Year	*N* BHSP collected females	*N* eggs collected from BHSP	*N* viable adults produced
1	Feb/ 03	2	8	2
2	Mar/Apr 03	4	30	65
3	May 03	2	31	192
4	Jun 03	2	31	356
5	Jul/Aug 03	0	0	623
6	Aug/Sept 03	0	0	1,411
7	Sept/Oct 03	0	0	1,274
8	Oct/Nov 03	1	14	1,192
9	Nov/Dec 03	0	0	516
10	Dec 03 /Jan 04	0	0	297
11	Jan/Feb 04	0	0	253
12	Feb/Mar 04	0	0	589
13	Mar/Apr 04	0	0	1,488
14	Apr/May 04	0	0	1,378
15	Jun 04	0	28	923
16	Jun/Jul 04	0	0	1,234
17	Aug 04	0	0	1,078
18	Aug/Sept 04	0	0	1,345
19	Sept 04	0	0	1,234
20	Oct 04	0	0	1,647
21	Nov 04	0	0	671
22	Dec 04/ Jan 05	0	0	113
23	Feb/Mar 05	0	0	24
24	Apr/May 05	0	23	32
25	Jun 05	0	0	109
26	Jul 05	0	56	787
27	Aug 05	0	0	80
28	Sept 05	0	0	87
29	Oct/Nov 05	0	0	54
30	Dec 05	0	0	63
Total		11	221	19,119

Table 5-2. Summary of second iteration captive colony numbers of *C. t. bethunebakeri* from April 2006 to December 2007. *N*, number of (Bahia Honda State Park) BHSP collected females refers to those from which eggs originated and were incorporated to the captive colony; number of eggs collected from BHSP were directly collected from BHSP and added to the captive colony.

Captive generation	Month/Year	*N* BHSP collected females	*N* eggs collected from BHSP	*N* viable adults produced
1	Apr/May 06	0	46	28
2	Jun 06	0	34	163
3	Jul 06	0	0	602
4	Aug/Sept 06	0	0	1,426
5	Sept/Oct 06	0	0	1,347
6	Nov/Dec 06	0	0	NA
6	Jan/Feb 07	0	0	NA
7	Mar/Apr 07	0	0	19
8	May/Jun 07	6	45	81
9	Jul 07	0	0	251
10	Aug 07	0	0	346
11	Sept 07	0	25	591
12	Oct 07	0	0	154
13	Nov/Dec 07	0	0	NA
Total		6	150	5,008

Table 5-3. Overview of molecular diversity measures for Bahia Honda State Park (BHSP) and captive colony (CC) populations of *C. t. bethunebakeri* (*N*). *n*, mean amplifiable samples across all microsatellite loci; N_A, observed number of alleles; n_e, effective number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; *, data pooled and analyzed as one population.

Population	*n*	N_A	n_e	H_O	H_E
BHSP 2005 (24)	23.750	4.417	2.068	0.365	0.457
BHSP 2006 (90)	86.833	7.000	2.246	0.403	0.494
BHSP Total (114*)	110.583	7.667	2.233	0.395	0.493
CC November 2005 (22)	19.500	4.750	2.776	0.373	0.573
CC June 2006 (15)	14.667	4.167	2.341	0.410	0.513
CC July 2006 (40)	38.667	4.667	2.045	0.392	0.460
CC October 2006 (10)	9.667	3.000	2.143	0.361	0.448
CC November 2006 (12)	11.750	2.583	1.705	0.297	0.343
CC 2004 (25)	24.083	4.167	2.062	0.332	0.427
CC 2005 (24)	21.500	4.750	2.751	0.373	0.569
CC 2006 (77)	74.750	6.083	2.128	0.377	0.475
CC Total (126*)	120.333	7.167	2.440	0.368	0.533

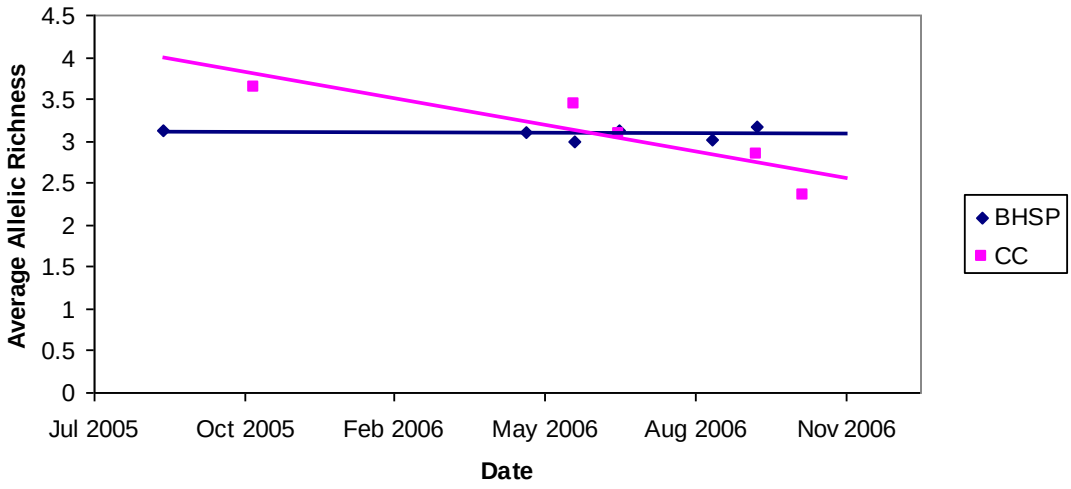


Figure 5-1. Average allelic richness values of 12 microsatellite loci measured in discrete generations of the Bahia Honda State Park (BHSP) and captive colony (CC) of *C. t. bethunebakeri*. Equations for the linear function trend lines are y = -6E-05x $^{+ 5.2469}$ and R^2 = 0.0119 (BHSP) and y = -0.0031x $^{+ 124.71}$ and R^2 = 0.7891 (CC).

Table 5-4. Effective population size (N_E) estimates of the 2006 captive colony of *C. t. bethunebakeri*. Results were generated by comparing allelic frequencies in consecutive generations in a moments based temporal model.

Generation	*N* generations	N_E	95% CI
June - July	2	14.8	6.7 - 47.5
October – November	2	3.7	1.7 – 9.3
June - November	6*	6.9	4.7 – 11.3

* The total number of elapsed generations June-November is six, real data exists only for 4 generations as there are no data for August or September 2006.

Table 5-5. Pairwise population matrix of Nei's genetic distances (D_S). Bahia Honda State Park (BHSP) and captive colony (CC) populations of *C. t. bethunebakeri*.

BHSP Sept 2005	BHSP May 2006	BHSP June 2006	BHSP July 2006	BHSP Sept 2006	BHSP Oct 2006	CC Nov 2005	CC June 2006	CC July 2006	CC Oct 2006	
0.033										BHSP May 2006
0.050	0.032									BHSP June 2006
0.074	0.061	0.044								BHSP July 2006
0.077	0.099	0.075	0.068							BHSP Sept 2006
0.097	0.084	0.088	0.088	0.112						BHSP Oct 2006
0.138	0.117	0.158	0.131	0.192	0.113					CC Nov 2005
0.078	0.050	0.051	0.054	0.139	0.125	0.116				CC June 2006
0.087	0.054	0.042	0.066	0.130	0.112	0.119	0.038			CC July 2006
0.142	0.098	0.096	0.102	0.199	0.165	0.182	0.075	0.058		CC Oct 2006
0.156	0.118	0.112	0.106	0.196	0.102	0.170	0.116	0.080	0.098	CC Nov 2006

Table 5-6. Pairwise population matrix of Nei's unbiased genetic distances (unbiased D_S). Bahia Honda State Park (BHSP) and captive colony (CC) populations of *C. t. bethunebakeri*.

BHSP Sept 2005	BHSP May 2006	BHSP June 2006	BHSP July 2006	BHSP Sept 2006	BHSP Oct 2006	CC Nov 2005	CC June 2006	CC July 2006	CC Oct 2006	
0.012										BHSP May 2006
0.035	0.014									BHSP June 2006
0.039	0.023	0.013								BHSP July 2006
0.041	0.061	0.043	0.016							BHSP Sept 2006
0.065	0.049	0.059	0.039	0.063						BHSP Oct 2006
0.113	0.089	0.136	0.089	0.150	0.074					CC Nov 2005
0.050	0.019	0.026	0.009	0.094	0.083	0.080				CC June 2006
0.072	0.036	0.031	0.034	0.098	0.083	0.097	0.014			CC July 2006
0.110	0.063	0.068	0.053	0.150	0.119	0.143	0.033	0.030		CC Oct 2006
0.135	0.094	0.094	0.068	0.158	0.067	0.142	0.086	0.062	0.063	CC Nov 2006

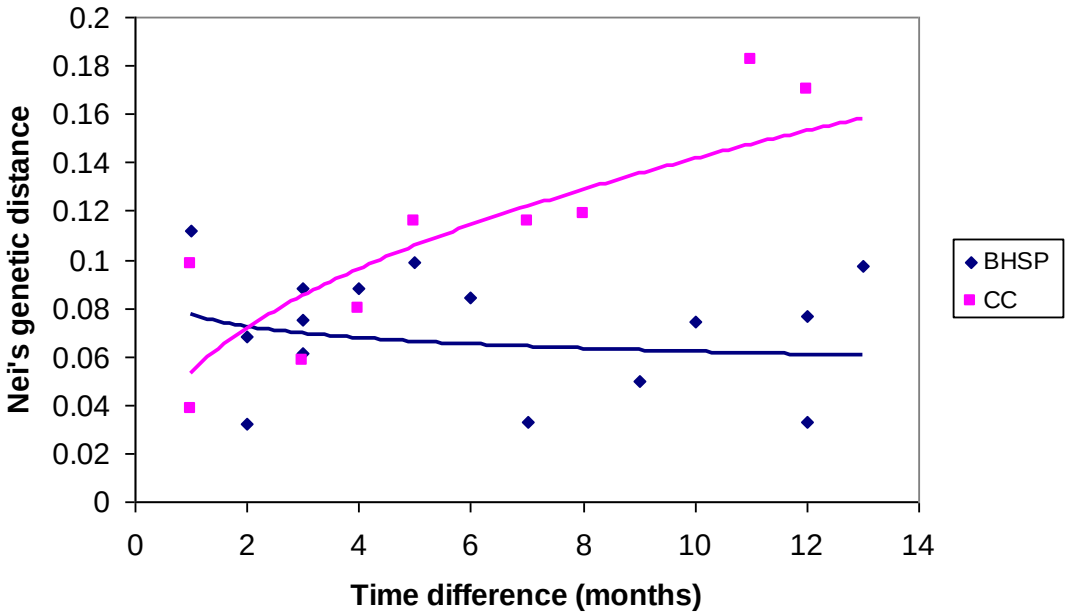


Figure 5-2. Nei's genetic distance between Bahia Honda State Park (BHSP) and captive colony (CC) butterflies over time. Equations for the power function trend lines are $y = 0.077x^{-0.0971}$ and $R^2 = 0.0328$ (BHSP) and $y = 0.0532x^{0.4248}$ and $R^2 = 0.6304$ (CC).

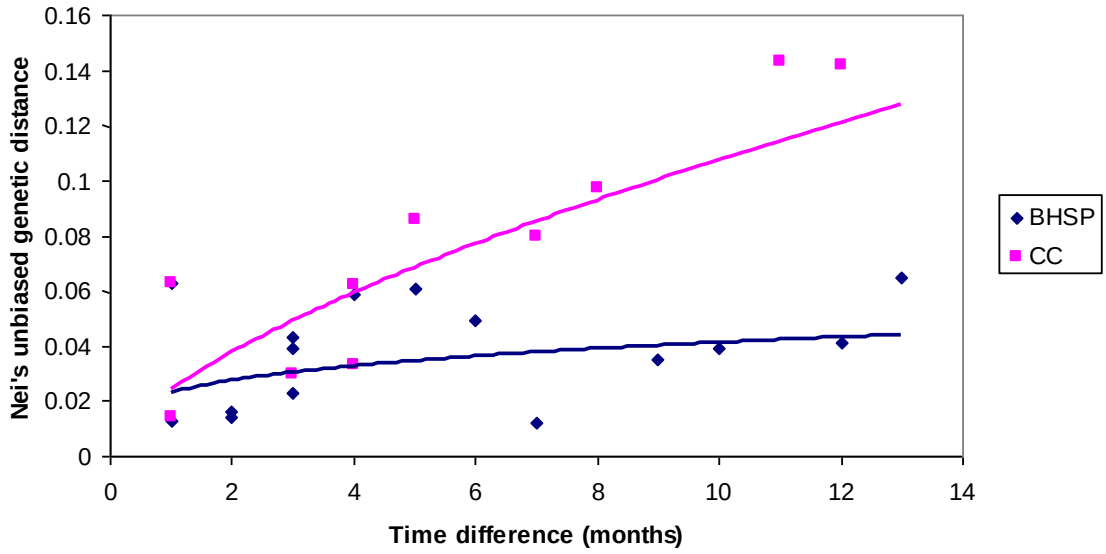


Figure 5-3. Nei's unbiased genetic distance between Bahia Honda State Park (BHSP) and captive colony (CC) butterflies over time. Equations for the power function trend lines are $y = 0.023x^{0.2524}$ and $R^2 = 0.1184$ (BHSP) and $y = 0.024x^{0.6509}$ and $R^2 = 0.6035$ (CC).

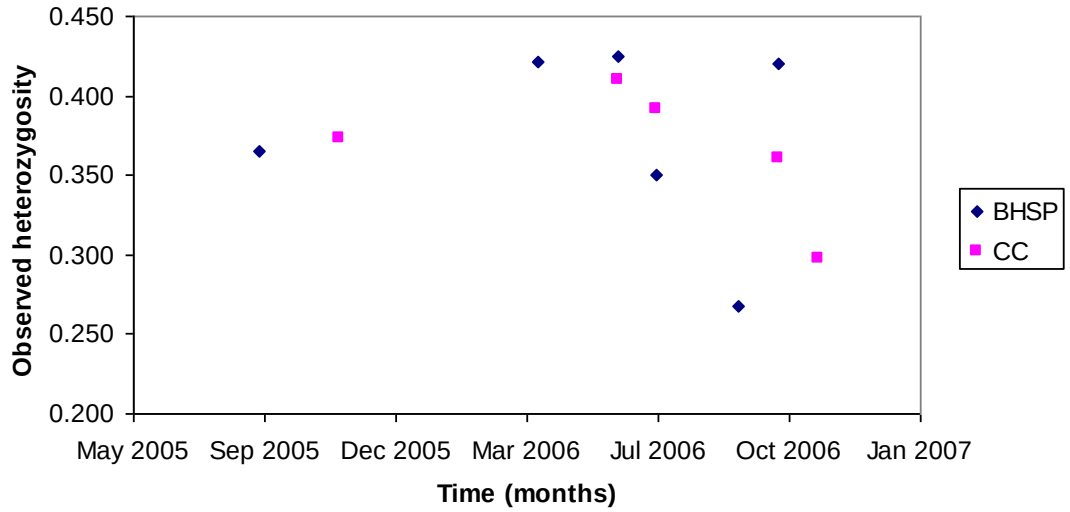


Figure 5-4. Observed heterozygosity values averaged over 12 microsatellite loci for generations of *C. t. bethunebakeri*; BHSP, Bahia Honda State Park population; CC, captive colony.

Table 5-7. Wilcoxon signed rank tests for excess (top) and deficiency (bottom) of heterozygosity for populations of captive-raised laboratory populations of *C. t. bethunebakeri*; CC, captive colony. Significant values ($P < 0.05$) are in italics; IAM, infinite allele model; TPM, tow-phase model; SMM, stepwise mutation model.

Population	Sample size	IAM	TPM	SMM
CC-June-2006	15	0.455/0.575	0.830/0.190	0.993/*0.017*
CC-July-2006	40	0.661/0.367	0.979/*0.026*	0.999/*0.001*
CC-October-2006	10	*0.003*/0.998	0.103/0.913	0.289/0.740
CC-November-2006	12	0.150/0.875	0.327/0.715	0.545/0.500

CHAPTER 6
THE GENETIC AND FITNESS CONSEQUENCES OF FOUNDER EFFECTS ON LABORATORY POPULATIONS

Introduction

Small population size, bottleneck events, and inbreeding go hand-in-hand yet are often treated separately in population-level studies. Bottlenecks result in reduced population size and can be caused by founder events, disease outbreaks, or habitat loss. Single-generation bottlenecks, known as founder events, lead to small populations with reduced genetic diversity and adaptive potential due to alleles lost as a result of the founder event (Frankham et al. 2003). Studies with model organisms have shown that not only are rare alleles lost in founder events but that overall allelic frequencies are distorted in the resulting populations (England et al. 2003). These populations will be of reduced size and subjected to elevated levels of genetic drift and inbreeding. Inbreeding depression often results in small populations and is realized as a loss of reproductive fitness, further contributing to population decline and extinction (Newman & Pilson 1997; Saccheri et al. 1998). This cascade of events is all initiated by founding populations with a few individuals, and is of great concern in both the management of endangered species populations and the creation of captive colonies.

Founder events are a severe form of bottleneck event (England et al. 2003) but are nonetheless a natural part of metapopulation dynamics (Hanski 1999; Haikola et al. 2004). Organisms that migrate to unoccupied habitat patches may found new colonies, often from single or very few individuals. In addition to environmental variables, the continued occupancy of such patches depends on the genetic variability of founders as well as that from new migrants. In many endangered taxa, however, there are few to no neighboring populations from which to receive new migrants. Newly founded populations are therefore dependent upon the genetic

variability inherent in its founding individuals and are subjected to high levels of inbreeding due to the limited mating options.

The Miami blue butterfly, *Cyclargus thomasi bethunebakeri*, is a Florida-listed endangered taxon that persists in a metapopulation structure at two locations in the lower Florida Keys; Bahia Honda State Park (BHSP) and Key West National Wildlife Refuge (FWC 2003; E.V. Saarinen, unpublished data). Previous research with this taxon has shown that gene flow is high between neighboring patches, but that butterflies do not readily colonize new patches further than 12m (J.C. Daniels, unpublished data). There is also no gene flow between the BHSP and KWNWR populations as they are over 70 km apart, separated by water barriers, and have no known intermediary, stepping-stone populations (FWC 2003; E.V. Saarinen, unpublished data.). Suitable habitat patches within the metapopulation frameworks are regularly colonized, go extinct, and are subsequently re-colonized by successive generations of butterflies. Habitat patches may be colonized by single gravid females or by multiple immigrants.

The goals of this study are to assess the effect of founder events on various levels of genetic diversity with the ultimate goal of evaluating evolutionary potential of a population after such an event. In order to investigate the fitness and genetic consequences of new colony founding, I established artificial laboratory founder lines of butterflies so as to mimic the beginning of new populations by a limited number of adults. I then measured fitness in the resulting generation of butterflies and time to extinction of each of the populations. I used heterozygosity and other molecular measurements to assess genetic variability in newly founded laboratory populations.

Methods

Experimental Treatment Lines

One pair founder lines

I created experimental lines of butterflies originating from wild-caught BHSP females from 2005-2007. Females were only removed from BHSP when field numbers were high and in compliance with FWC permits. When BHSP population estimates were low, experimental lines were founded from individuals from an already-established University of Florida captive colony. In these instances, only organisms that had been in captivity for one generation were used to found experimental lines. All female butterflies were kept alive in individual oviposition cups and supplied with ample artificial nectar (Gatorade® on a cotton swab) and fresh terminal growth of host plant *Caesalpinia bonduc* (L.) Roxb. (Fabaceae). As all wild-caught and captive-colony females were assumed to be gravid, I stimulated oviposition with an alternating light regime in the laboratory. Eggs from these females were kept separately and the resulting larvae were allowed to complete development. As adult eclosions occurred, full-sibling individuals were moved to individual flight cages outside of the McGuire Center for Lepidoptera and Biodiversity, Gainesville, Florida (FLMNH) and allowed to mate. Only full-siblings were present in each flight cage. All flight cages were monitored throughout the day at regular intervals of 15 minutes between 9am and 6pm and recorded mating pairs of butterflies were removed. All butterflies used in this study mated for at least 45 minutes and, after natural separation, females were placed in individual oviposition cups with ample artificial nectar (Gatorade® on a cotton swab) and fresh terminal growth of *C. bonduc*. Males were not returned to the flight cage. The eggs that resulted from each full-sibling-mated female were removed and maintained in individually labeled containers. In this manner, the developing larvae, pupae, and adults that resulted from each full-sibling mating were maintained as discrete groups. Each

group was supplied with ample host plant and conditions were identical to those for raising the captive colony. Twelve replicates of single-founder lines were completed in 2005-2006.

Three pair founder lines

Three pairs of unrelated females and males (all from the University of Florida captive colony) were introduced to individual flight cages for the establishment of three pair experimental founder lines. Cages were regularly monitored and matings by the three females in each replicate were verified. The population of each line was again given ample food at each life stage. Lines founded by three pairs of adults were replicated three times in 2007.

Control Groups

Butterflies from the University of Florida captive colony were used as the control group for comparison with the different artificial founder-line treatments as it was not possible to track individuals in the BHSP wild population. During the time of the experiments, the captive colony was regularly-infused with new genetic material from the wild BHSP population, ensuring its status as an outbred population.

Fitness Measurements and Statistical Analyses

Measurements of fitness were recorded from individuals in all experimental treatments as well as from a subset of individuals from the University of Florida captive colony (control group). Fitness measurements and genetic samples were taken from a random sampling of individuals in the captive colony. All measurements were taken during the same time period that the treatment experiments were conducted, ensuring that larvae and adults were raised under identical environmental conditions and given host material of similar quality and from the same source.

Life history traits typically show greater inbreeding depression than morphometric traits (Coltman & Slate 2003), and the former were measured in each generation. I counted the

number of eggs that each female butterfly laid for the duration of her lifetime in each population of treatment and control butterflies. I tested the number of days over which females laid their eggs to account for any differences caused by long-lived females in control and treatment groups. I also recorded the number of adults that successfully eclosed in each population. To account for differences in the starting number of eggs from each female, this statistic was converted to a ratio of the number of adults that successfully eclosed from the starting number of eggs. Egg-laying and viability is a well-documented fitness measurement for Lepidoptera (Saccheri et al. 1996) and the ratio of successfully-eclosed adults generated from initial eggs encompasses the entire life cycle. The variance of each population was tested with an *F*-test and Levene's test to assess differences between treatment and control groups. Statistical tests (2-sample *t*-tests assuming unequal variances when appropriate) compared the means of the one pair founder line populations and concordantly-run controls, the three pair founder lines and their respective controls, and the pooled treatments vs. the pooled controls. All tests were performed using Minitab® statistical software, version 13.32 (http://www.minitab.com).

DNA Extractions and PCR Amplifications

I developed a relatively non-invasive method to assess molecular diversity in *C. t. bethunebakeri* in all experimentally-founded lines as well as from individuals from the University of Florida captive colony. I gently held and removed a 2 mm^2 fragment of each butterfly's hind wing with a pair of forceps. The removed portion of the wing was placed directly in a vial with 90-100% ethanol and I carefully cleaned the forceps to prevent cross-contamination between samples. Sample vials were kept in a cool, dark place until they could be placed in a -80℃ freezer for storage. I extracted DNA from butterfly wing fragments using Qiagen DNeasy Blood and Tissue Kits (Qiagen©, USA). I followed the manufacturer's protocols for kit extractions with minor modifications owing to the small size of the wing

material I used for extraction. I macerated wing material with sterile, disposable micro-pestles and performed a final one-time elution of each DNA template in 50µL of warm 10mM TRIS. I stored DNA extractions at -20°C until I proceeded with PCR amplifications. All PCR reactions follow the protocols outlined in Saarinen et al. (2009) for amplification of 12 polymorphic microsatellite loci. All private alleles were individually assessed for validity and that they were not the result of genotyping or scoring error.

Genetic Statistical Analyses

Analyses of observed and expected heterozygosity and effective number of alleles were assessed using GenAlEx 6.1 (Peakall & Smouse 2006). I used FSTAT version 2.9.3.2 (Goudet 2001) to assess gene diversity and allelic richness in control and treatment groups of butterflies. Allelic richness is one measurement of diversity unbiased by sample size; this measurement compares the number of alleles per population and allows for sample sizes to be different. Gene diversities were estimated at each locus and treatment using Nei's unbiased estimator (Nei 1987) and were then averaged across the twelve loci for each population as recommended by England et al. (2003). Differences in gene diversity and allelic richness between treatment and control groups were assessed by ANOVA tests.

The loss of heterozygosity after a founder effect was calculated with the equation

$$\Delta H = H_1 - H_0 \qquad (6\text{-}1)$$

where H_1 is the heterozygosity after the founder event, H_0 is the heterozygosity before, and ΔH is the difference in the two generations. The empirical loss of heterozygosity was compared with the expectation that 25% of heterozygosity will be loss after a founder event of two individuals (England et al. 2003; Frankham et al. 2003).

Results

The results from these studies show that full-siblings of *C. t. bethunebakeri* will mate with each other and produce viable offspring. Additionally, observations of the mating cages did not indicate any full-sibling mating avoidance behaviors. However, because of other demographic factors (presence of a baculovirus that killed many larvae, failure for males and females to emerge at overlapping times, etc.), all lines of one pair and three pair founded populations went extinct after one round of full-sibling mating.

There was no difference in the number of days over which any females (of any treatment or control groups) laid eggs ($F_{1,32} = 1.448$, $P = 0.238$). Populations that were founded by one pair of butterflies did not show a reduction in the number of eggs laid per female when compared to the outbred captive colony ($T = -1.08$, df = 20, $P = 0.294$) (Table 6-1). Populations that were founded by three pairs of butterflies did show a reduction in the number of eggs laid per female ($T = -2.71$, df = 17, $P = 0.015$). When the one and three pair founder lines were pooled and compared against the control groups, there was a reduction in egg laying in the treatment group ($T = -2.70$, df = 37, $P = 0.010$). The lifetime fitness measure of the number of adults surviving from eggs was compared and all treatment groups were significantly lower than the respective control groups. One-pair founded populations showed reduced adult/egg ratios ($T = -2.57$, df = 8, $P = 0.033$). Three-pair founded populations were also reduced when compared to their controls ($T = -4.70$, df = 9, $P = 0.001$) as were the pooled founder number treatments vs. pooled controls ($T = -4.86$, df = 20, $P = 0.000$).

After one generation of full-sibling inbreeding (founding by a single pair of adults) there was no significant difference in gene diversity ($P = 0.413$) or allelic richness ($P = 0.080$) with an outbred population (Table 6-2). Heterozygosity measures were consistent, showing no significant differences in observed heterozygosity in samples from populations founded by one

pair of adults vs. samples from an outbred population ($P = 0.084$). Although these results did not show significant differences between one pair founded populations and the outbred individuals, allelic frequencies still changed after one round of inbreeding (Figures 6-1, 6-2, 6-3). These three microsatellite loci (CthB119, CthC127, and CthD7) are indicative of other microsatellite loci and showed how low frequency alleles are lost after a founder event and how high-frequency alleles may become fixed. No statistics were calculated for the three pair treatments as only one adult butterfly was produced from the three replicates.

These results show a decrease in heterozygosity of 15.8% after a new population was founded by one pair of butterflies versus an outbred population. These results are not as severe as the expected value derived from the hypothesis that a 25% of loss of heterozygosity per generation will result from a population founded by only two reproducing individuals (Frankham et al. 2003).

An unexpected result of this experiment was the discovery of a baculovirus affecting the larvae of *C. t. bethunebakeri*. Larvae from both experimental treatments (one pair and three pair founded populations) were observed to become slow-moving, paler in color, and the appearance of a yellow saddle was evident in the middle of the larvae (Figure 6-4). All larvae with these symptoms died within 24 hours, and larvae raised in the same cups as infected larvae also became afflicted. Identification of the baculovirus was performed by Drs. James and Aléjandra Maruniak, virologists at the University of Florida. Several larvae in the captive colony (2007) also became symptomatic of the virus, and measures were quickly taken to quarantine these individuals and to bleach the laboratory to prevent further spread of the virus. As both treatment and control individuals were affected, infection with this virus is not assumed to be due to reduced viral resistance in inbred individuals.

Conclusions

Experimental Shortcomings and Fortuitous Discoveries

This study affirmatively showed a decrease in fitness measures between populations founded by reduced numbers of individuals vs. the outbred group, but was unable to distinguish any difference between one pair or three pair founded populations because of poor sample size in the latter group. It is unclear why only one adult successfully eclosed from the three replicates of three pair founded populations. Sixteen butterflies eclosed from the 12 replicates of one pair founded populations.

This study also revealed larval death from baculovirus infection. This infection occurred in experimental lines founded by one pair of butterflies in 2005 and 2006 as well as in all of the three pair founded populations in 2007. Individuals from the 2006 and 2007 captive colony also had individuals afflicted with the baculovirus and as such, a direct correlation between inbreeding and lack of resistance to this virus cannot be inferred. Other insect conservation captive-breeding programs have had problems with pathogens (Cunningham & Frank 1993; Pearce-Kelly et al. 1998; Mattoni et al. 2003) but this is the first time a baculovirus has affected an endangered Lepidoptera captive breeding colony. Knowledge of the virulence of this pathogen has led to greater monitoring and prevention regimes in the management practices of the captive colony.

An additional piece of ecological knowledge was gained insofar as I observed no avoidance of full-sibling mating. These results are concordant with those found in the Glanville fritillary butterfly (*Melitaea cinxia*) (Haikola et al. 2004). In a small population, not mating with close relatives likely means not mating at all and therefore not reproducing. Thus, it is not surprising that both *M. cinxia* and *C. t. bethunebakeri*, two taxa that exhibit metapopulation structuring with small populations colonized by low numbers of founders, have not developed

strategies to avoid mating with siblings. Further knowledge of the ecology of *C. t. bethunebakeri* shows that adults are poor dispersers (J.C. Daniels, unpublished data) and are as such unable to migrate to distant habitat patches to mate with non-relatives. Studies of model organisms have shown that mating with relatives has likely led to the purging of deleterious alleles in homozygotes (Charlesworth & Charlesworth 1987; Crnokrak & Barrett 2002). Although still preliminary, this study suggests that the inability to detect or failure to refuse close siblings as mates may actually help save endangered taxa such as *C. t. bethunebakeri* and may have led to the purging of deleterious alleles.

Founder Effects on Heterozygosity, Genetic Diversity, and Evolutionary Potential

Work by Frankham et al. (1999) and England et al. (2003) have shown how population bottlenecks affect the evolutionary potential and short-term adaptability of populations of *Drosophila melanogaster*. Furthermore, these studies showed how, in the short-term, allelic diversity is lost rather than heterozygosity. In the current study, significant amounts of heterozygosity and allelic diversity were actually retained in bottlenecked populations, but the sample sizes of these studies were small and grand inferences cannot be drawn. Additionally, this study was successful only over one generation, whereas other studies using a model organism included data from multiple generations. This study is, however, important in that it showed how an endangered taxon reacts to experimental founding in the laboratory; data that are directly applicable to reintroductions in the field as well as to the beginnings of new populations for captive-breeding.

Severe bottlenecks result in an immediate loss of rare alleles, causing a reduction in allelic diversity faster than loss of heterozygosity (Nei et al. 1975; Allendorf 1986). Results from this study support the previous statement, showing that rare alleles were immediately lost and that heterozygosity decreased by 15.8% after a new population was founded by one pair of butterflies

versus the outbred control. These results were not as severe as the expected value derived from the hypothesis that a 25% of loss of heterozygosity per generation will result from a population founded by only two reproducing individuals (Frankham et al. 2003). However, this butterfly exhibits a generation time of approximately one month (FWC 2003) and within several months a large amount of genetic variability and evolutionary potential could easily be lost.

Results from this study support the idea that a bottleneck (single-generation founder event) does not cause an immediately significant reduction in heterozygosity. Prolonged reduction in population size will have this effect (Frankham et al. 2003). Results from the current study do show how rare alleles are lost after founder events, resulting in lower allelic richness, but not a significant reduction after one generation. The distribution of microsatellite alleles changes after a founder event and low frequency alleles are lost. Individual alleles are the source of evolutionary potential and have been shown to be important in insecticide and disease resistance (McKenzie & Batterham 1994; England et al. 2003). However, in mutation-selection balance, there is an equilibrium between losing rare alleles that are fuel for evolutionary adaptability and losing alleles that are neutral or slightly deleterious (Kimura 1983). In small populations, such deleterious alleles will be lost and result in a population with lower genetic diversity but higher fitness than a larger, more outbred population.

In a 2003 meta-analysis, Reed and Frankham show that population genetic diversity is highly correlated with population fitness, reinforcing the idea that the loss of heterozygosity has a detrimental effect on a population. Because of the link between heterozygosity and population size (Frankham 1996; Frankham et al. 2001), heterozygosity can provide insight into a population's fitness, even if heterozygosity measures are taken from neutral molecular markers. However, this study has shown not only how founder events may produce populations of

significantly reduced fitness but that fitness declines may be observed before decreases of neutral genetic diversity can be detected. It is therefore important to monitor both direct measurements of fitness as well as neutral genetic diversity as the latter will not as accurately predict pending population crashes. Considering the forces (genetic drift and inbreeding) that impact small populations is important in endangered species management as these factors lead to both heterozygosity and fitness declines (Frankham et al. 1999). Populations founded by low numbers of individuals become extinct at higher levels, thus denoting the importance of avoiding population bottlenecks in species of conservation concern.

Table 6-1. Effects of founder lines on fitness measures (mean ± standard error). There were no significant differences between the one pair and three pairs treatments for either variable and there were no significant differences between controls.

Treatment	Replicates	Eggs laid/female	Adults/eggs
1-pair	12	77.583±21.859	0.035±0.024
1-pair control	8	114.000±26.107	0.199±0.059
3-pairs	3	61.230±20.515	0.001±0.001
3-pairs control	10	154.500±27.665	0.163±0.035

Table 6-2. Effects of founder lines on genetic variability measures. *N*, sample size of butterflies genotyped; N_A, total number of alleles; N_E, effective number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity. The three pair treatment resulted in only one female butterfly.

Treatment	*N*	N_A	N_E	H_O	H_E	Genetic diversity	Allelic richness
1-pair	14.917	2.917	1.783	0.234	0.391	0.411	2.797
1-pair control	40.667	4.750	2.094	0.392	0.470	0.477	3.619
3-pairs	0.917	1.083	1.083	0.167	0.083	NA	NA
3-pairs control	8.750	3.500	2.439	0.415	0.529	0.572	3.223

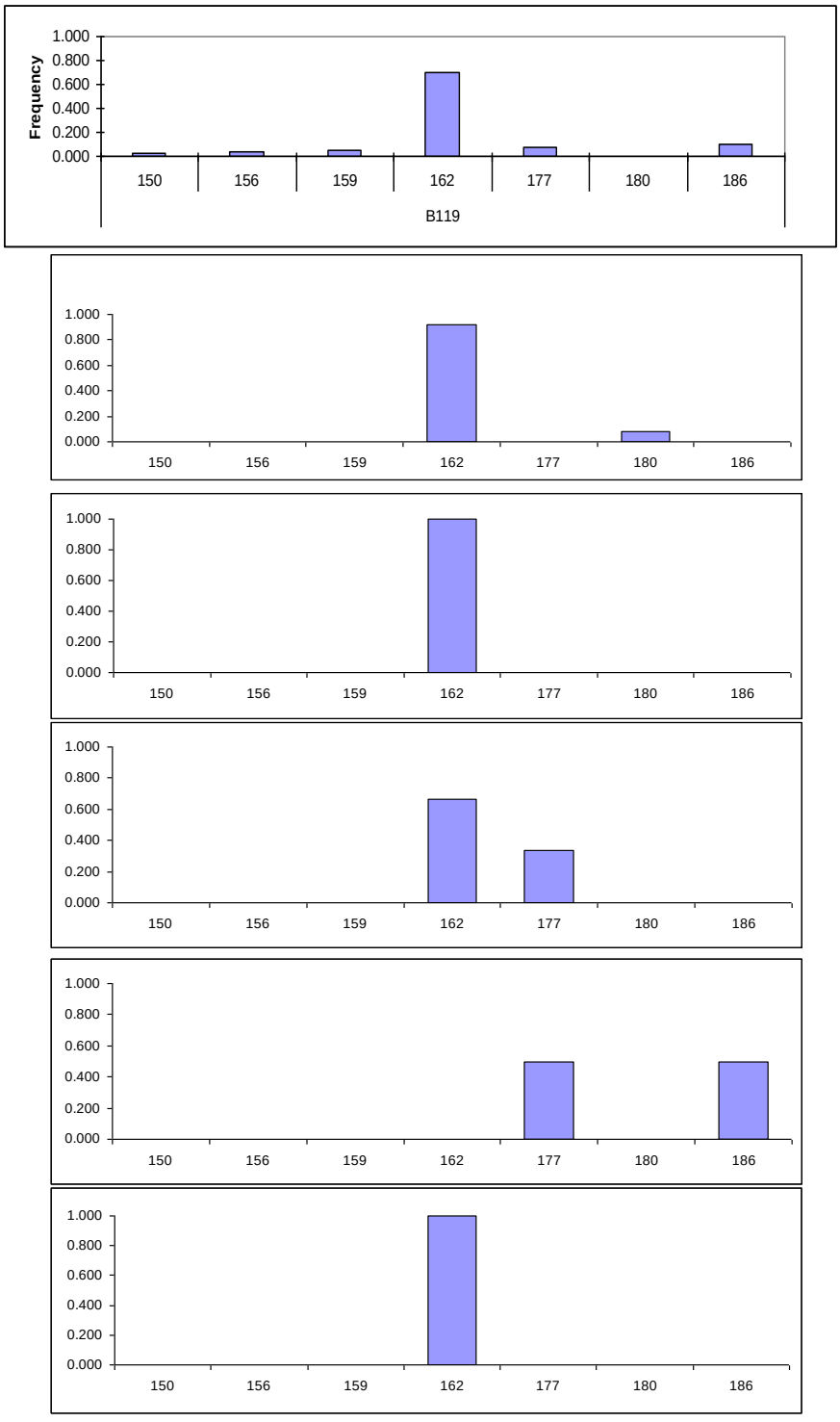


Figure 6-1. Distributions of alleles (bp) at locus CthB119 in an outbred population (top) and populations founded by one-pair of adult *C. t. bethunebakeri* (five replicates).

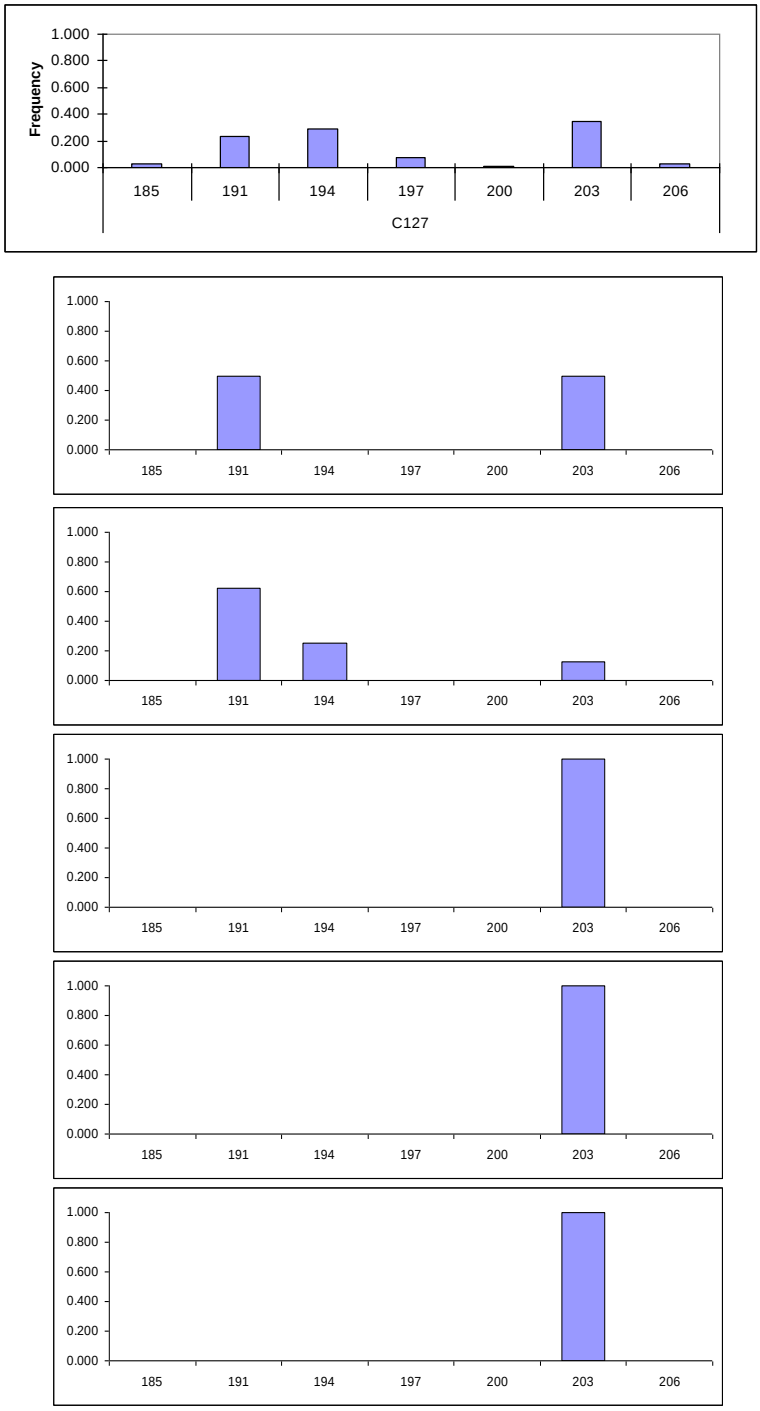


Figure 6-2. Distributions of alleles (bp) at locus CthC127 in an outbred population (top) and populations founded by one-pair of adult *C. t. bethunebakeri* (five replicates).

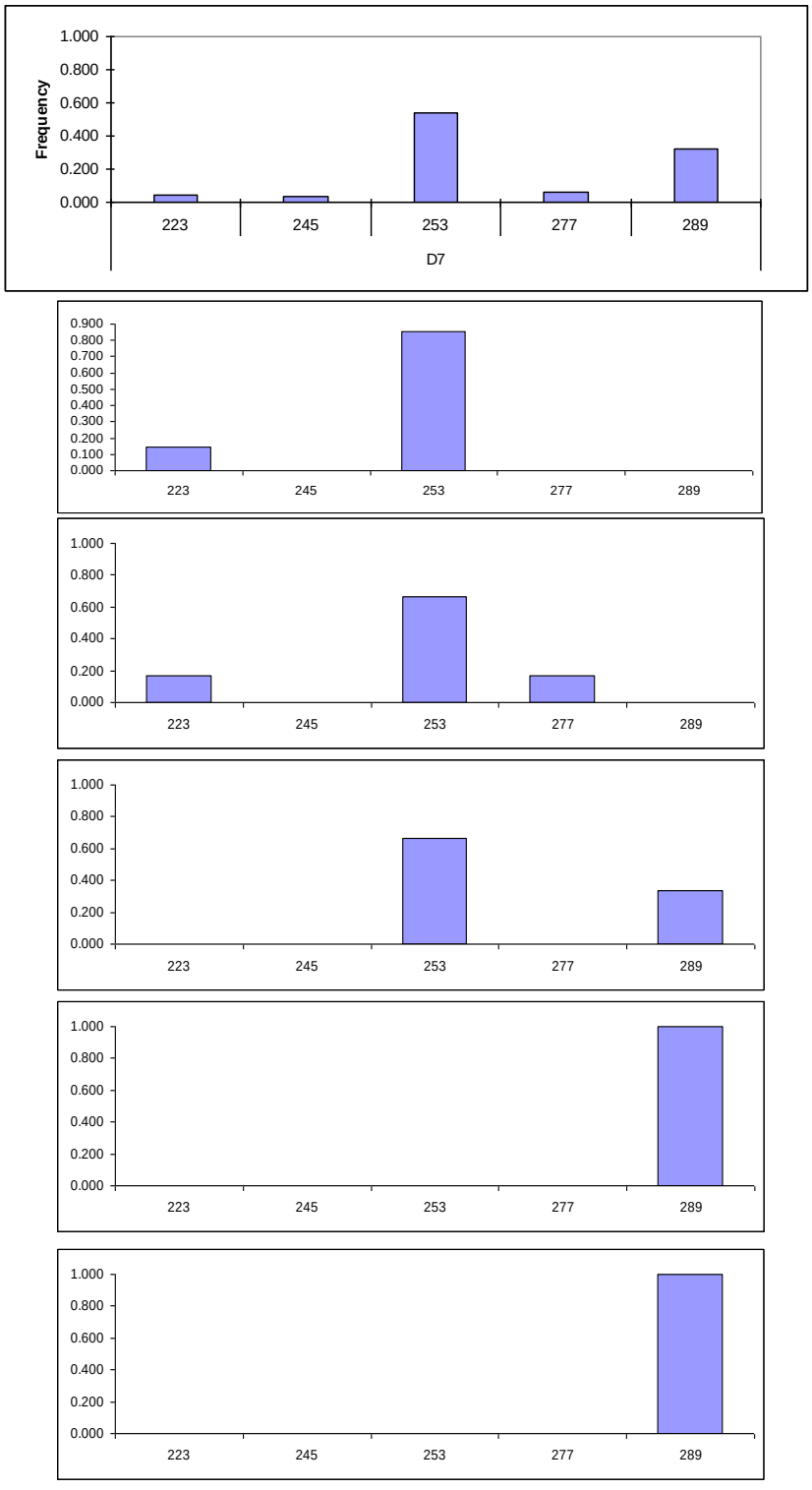


Figure 6-3. Distributions of alleles (bp) at locus CthD7 in an outbred population (top) and populations founded by one-pair of adult *C. t. bethunebakeri* (five replicates).

Figure 6-4. *Cyclargus thomasi bethunebakeri* larvae killed by baculovirus.

CHAPTER 7
SYMBIOSIS BETWEEN MIAMI BLUE LARVAE AND ANTS

Introduction

Over 75 percent of lycaenid larvae with known life histories associate with ants (Pierce et al. 2002). Such myrmecophilous relationships may be mutualistic to varying degrees or even parasitic whereby larvae are predatory in ant nests (Pierce & Mead 1981; Fiedler & Maschwitz 1988; New 1993). The resulting communication between larvae and ants is mediated by a complex array of tactile, chemical, and often audible signals (DeVries 1990). Specifically, larvae possess highly specialized organs that can extrude alarm, reward or appeasement chemicals. In response, tending ants often protect the surrounding larvae from a variety of natural predators and parasitoids, and thus can potentially provide a benefit for survival (Thomas 1980; Webster & Nielson 1984; Pierce & Easteal 1986; Savignano 1994). The presence of an ant guard has also led to larger adults in the related butterfly *Hemiargus isola* (Reakirt). When larvae are reared with ants they may expend less energy than when they are untended and therefore develop into larger potentially more fecund adults (Wagner & Martinez del Rio 1997). Cushman & Murphy (1993) suggest that ant associations may also play an important role in the persistence of lycaenid populations. They additionally propose that species with a dependence on ants, whether facultative or obligatory, display an increased sensitivity to environmental change, and thus are more susceptible to endangerment than species that lack ant associations.

The Miami blue, *Cyclargus thomasi bethunebakeri* (Comstock & Huntington) (Lycaenidae), represents one of Florida's rarest endemic butterflies and is currently listed as state-endangered. Once commonly found in tropical coastal hammocks and their associated margins, beachside scrub and tropical pine rocklands from the southern Florida mainland south through the Florida Keys to Key West and the Dry Tortugas, the species' overall distribution and

numerical abundance has now been reduced to two remaining metapopulations within the boundaries of Bahia Honda State Park in the Lower Keys and the islands of Key West National Wildlife Refuge (Klots 1964; Kimball 1965; Lenczewski 1980; Minno & Emmel 1993; Ruffin & Glassberg 2000; Calhoun et al. 2002; FWC 2003). Developing larvae of *C. thomasi bethunebakeri* have been shown to be tended by ants in the genus *Camponotus* but the extent of the relationship has remained poorly understood (Minno & Emmel 1993). Recent population studies of the butterfly at Bahia Honda State Park and additional reintroduction sites within Everglades National Park confirm a continued association. Here, I provide a more detailed account of this relationship, identify additional ant associates and potential predatory ant species, and discuss the corresponding implications for the conservation and recovery of this critically imperiled butterfly.

Methods

Field surveys of ant species' composition were conducted at Bahia Honda State Park and the Flamingo Campground, Rowdy Bend Trail, and Bear Lake Road sites in southern portions of Everglades National Park during daylight hours on 24-27 May 2004 and 31 July - 2 August 2004. These areas contain small numbers of *C. thomasi bethunebakeri*, either as part of the remaining natural metapopulation or as relocated individuals. Ants were surveyed on and around *Caesalpinia bonduc* (L.) Roxb. (Fabaceae), the current primary larval host plant of the Miami blue, by extensive hand-collecting and baiting. Sugar baits consisting of a series of at least ten index cards each with approximately 10g of crushed pecan cookie, were placed along a transect passing along the base of *C. bonduc* plants. Baits were left out for one hour, at which time the resulting cards with ants were collected in Ziploc-style plastic bags. Additionally, when larvae of *C. thomasi bethunebakeri* were found in association with ants, 1-2 ant specimens were

collected from the tended larvae. This research was conducted under permits EVER-2004-SCI-0038 and 5-04-58 for Everglades National Park and Bahia Honda State Park.

Finally, to provide additional detail on the structure of the larval ant organs, three *C. thomasi bethunebakeri* larvae from the captive colony maintained at the University of Florida were preserved and used for SEM and Auto-Montage photographic analysis. Larvae were placed in near boiling water for 60 seconds, transferred to 25% ethanol for two hours, 50% ethanol for another two hours, and stored in 75% ethanol before they were photographed.

Results

A total of 18 ant species were collected in Everglades National Park and Bahia Honda State Park and results are summarized in Table 7-1. Of the 18 collected species, five were confirmed tending larvae of *Cyclargus thomasi bethunebakeri*: *Camponotus floridanus, Camponotus planatus, Crematogaster ashmeadi, Forelius pruinosus,* and *Tapinoma melanocephalum. C. floridanus* were found tending larvae every time larvae were encountered in the field when other ants were not present. All other ants were encountered 1-2 times in the field, and no two species of ants tended larvae at the same time. There were typically two ants tending a larva at a time, with the exception being *Crematogaster ashmeadi,* which tended larvae in higher numbers (Figure 7-1).

An additional two species, *Paratrechina longicornis* and *Paratrechina bourbonica* have been named as potential ant associates. The former species has been found in proximity to *C. thomasi bethunebakeri* larvae and appears to tend them, although encounters are brief. The latter species was observed tending larvae of another lycaenid, *Strymon martialis* (Herrich-Schäffer), on *Caesalpinia bonduc* at Bahia Honda State Park. No predation by these ants was observed in the field.

Details of the ant organs of *Cyclargus thomasi bethunebakeri* are shown in Figure 2. Second through fourth instars possess a dorsal nectary organ (=honey gland) with associated pore cupolas (=lenticles) on A7 and a pair of eversible tentacle organs on A8. Abdominal segments A7 and A8 are fused dorsally. Tentacle organs were observed to evert independently in the field when stimulated by attendant ants and liquid droplets from the honey gland were actively imbibed by all species of ants. *Camponotus floridanus* became excited and agitated, evidenced by increased body and antennal movements, when the tentacle organs were everted.

Discussion

This study definitively finds *Camponotus floridanus* as the species in primary attendance of the endangered *Cyclargus thomasi bethunebakeri* larvae. *C. floridanus* is a native species occurring throughout Florida and is nocturnal; they are commonly found foraging on nickerbean and tending *C. thomasi bethunebakeri* larvae in both the Everglades and Bahia Honda locations. *Camponotus planatus* is a diurnal species, but is not commonly found tending larvae (it has never been found tending larvae in the Everglades and only once in Bahia Honda). This diurnal species may tend larvae when they are more abundant, and *C. planatus* may be important in protecting the species during the day. Buckley & Gullan (1991) have shown that more aggressive ants provide better protection for soft scales and mealybugs, and as a corollary, the large and potentially aggressive *Camponotus* species may prove effective in deterring predators and parasitoids from harming larvae (Axén 2000).

Crematogaster ashmeadi have been found in great numbers tending individual larvae in Bahia Honda but have not yet been observed in the Everglades. Again, these ants are not commonly found tending larvae, but when they are, there are at least five ants found, demonstrating the quality of their trailing and recruitment signals. Other *Crematogaster* species have been found worldwide to tend lycaenid larvae and this genus seems predisposed to lycaenid

symbioses (Atsatt 1981; Fiedler 1991; Pierce et al. 2002; Saarinen 2006). These ants are equipped with a flexible abdomen and attached sting; despite the small size they are capable of defending larvae from other ants or harmful invertebrates.

Both *Forelius pruinosus* and *Tapinoma melanocephalum* may be opportunistic ants imbibing food rewards from *C. thomasi bethunebakeri* larvae. Their behavior suggests no elevated protection for the larvae they tend; however, their presence may potentially deter predators. Both *Paratrechina longicornis* and *bourbonica*, along with *Tapinoma melanocephalum*, have been referred to as "tramp ants" (Passera 1994). While these tramp ants may not provide demonstrative protection of larvae, they may also defend the larvae as any other food source. At the very least, they tolerate these larvae and coincidentally tend instars that feed on nickerbean flowers and buds where the ants are also gathering nectar. In an assessment of potential ant partners, it was shown that *Tapinoma sessile* (Say) is a "neutral partner" for the widely distributed North American lycaenid *Glaucopsyche lygdamus* (Doubleday), providing no significant cost or benefit (Fraser et al. 2001). No ant association should be overlooked or underestimated, however. Researchers of the critically imperiled European lycaenid butterfly *Maculinea rebeli* (Hirschke) have repeatedly emphasized "the importance of identifying local host ant species prior to further management conservation strategies in order to avoid failure of management programs or even damage to populations on the edge of extinction" (Steiner et al. 2003).

The facultative nature of the ant associations are clear, encompassing four genera (five including *Paratrechina*) and three subfamilies; *Camponotus* and *Paratrechina* are Formicinae, *Crematogaster* is Myrmicinae, and *Tapinoma* and *Forelius* are Dolichoderinae. Larvae may exhibit "non-specific" ant semiochemicals as attractants to the various ant species, as proposed

by Henning (1983). These chemicals, primarily from the tentacle organs and potentially from the pore cupolas, may serve to either alarm, excite, or appease the ants. Further study into the chemical secretions of all organs, including the dorsal nectary organ but specifically of the tentacular organs, may clarify the "intentions" of the larvae in their emissions. Further comparisons of each ant species own alarm and attractant pheromones with those isolated from larval volatiles may further elucidate ant-larval relationships, including whether ants are specifically, chemically targeted and whether others are simply opportunistic tenders.

No interactions between the other identified ants and larvae were observed. Several of these ants, however, may be predating on the larvae at other times. Species of special concern include *Solenopsis invicta* Buren and *Wasmannia auropunctata* (Roger) in Bahia Honda State Park. All three *Pseudomyrmex* species may also potentially be predators, possibly excepting *P. simplex* (F. Smith) due to its small size. It is fortunate that in these locations, there are also abundant colonies of *Camponotus floridanus.* Further field observations, especially at night when *C. floridanus* are most active, need to be carried out to assess interactions of the ant mosaic when *C. thomasi bethunebakeri* larvae are present.

This study shows the persistence of *Wasmannia auropunctata* on Bahia Honda State Park (first recognized there by Deyrup et al. 1988). This invasive tramp ant is native to the New World tropics so its presence in the Florida Keys is not a surprise, but it may be a cause for concern. *W. auropunctata* also known as the little fire ant is an opportunistic feeder that forages day and night and that bears a painful sting. This ant has displaced several endemic invertebrates in the Galapagos Islands, and it has the potential to do so elsewhere (Meier 1994). It, along with the red imported fire ant *Solenopsis invicta,* is associated with a decline in endemic biodiversity (Meier, 1994; Wojcik 1994). Neither ant has been found near relocated colonies of *C. t.*

bethunebakeri in the Everglades nor has either ant been directly observed killing larvae or adults in Bahia Honda. *S. invicta* mounds are not as large or as extensive on Bahia Honda as they are in extremely disturbed habitats, and perhaps mound density is not large enough to impact the *C. t. bethunebakeri* metapopulations. Further field work will need to examine predation rates by ants, and the specific impact that these invasive ants may have on endemic butterfly species, especially species of special concern.

Figure 7-1. *Crematogaster ashmeadi* ants tending a late instar *Cyclargus thomasi bethunebakeri* larva. Several other *C. ashmeadi* ants were present but not visible in this photo. Reprinted with permission from Saarinen, EV and J.C. Daniels. 2006. Miami blue butterfly larvae (Lepidoptera: Lycaenidae) and ants (Hymepnoptera: Formicidae): new information on the symbionts of an endangered taxon. Florida Entomologist 89:69-74.

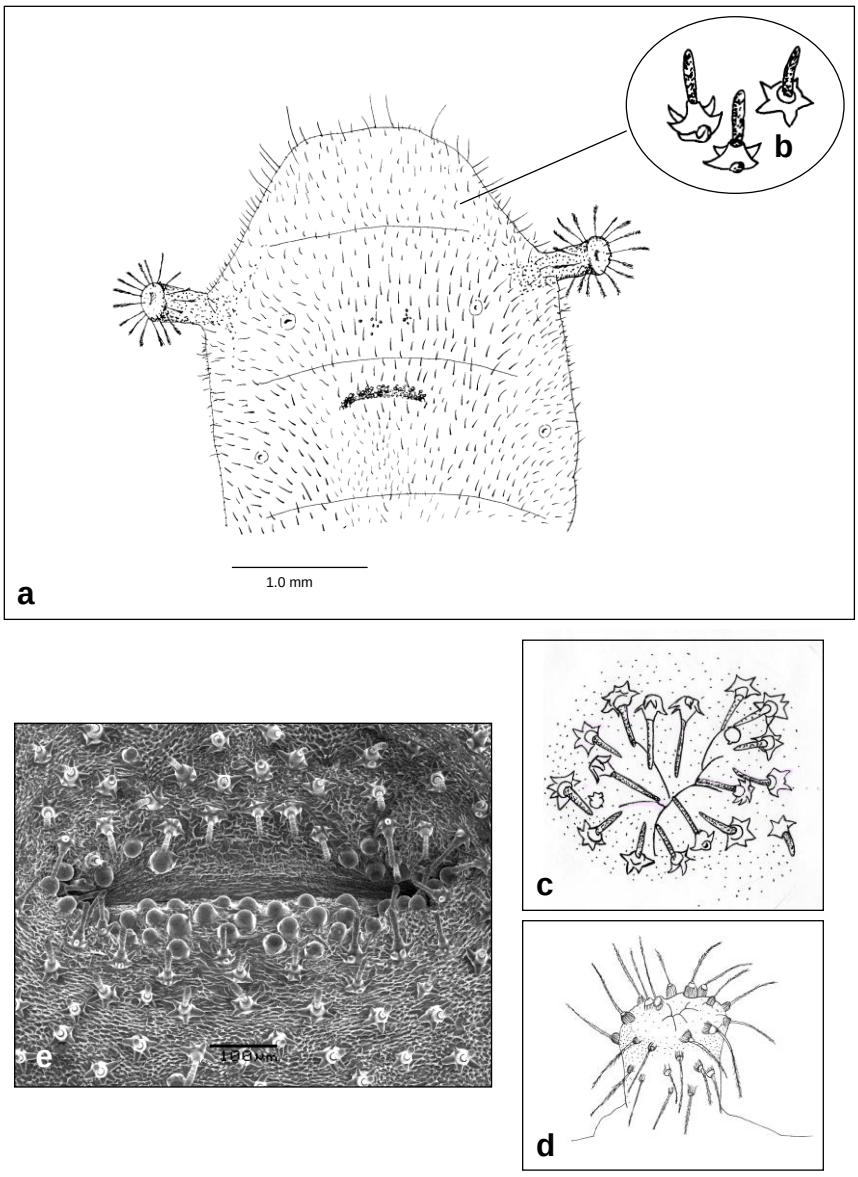


Figure 7-2. Details of *Cyclargus thomasi bethunebakeri* fourth instar caterpillar. a, dorso-posterior abdominal segments (A7-A8) showing ant organs; b, detail of cuticular setae; c, tentacle nectary organ inverted; d, tentacle nectary organ everted (cuticular setae omitted); e, dorsal nectary organ bordered by pore cupolas.

Table 7-1. Ants of Everglades National Park (ENP) and Bahia Honda State Park (BHSP) in Florida collected in proximity to larvae of the *C. t. bethunebakeri*. 1, ants collected by larvae in present study; 2, ants listed in Deyrup et al. (1988); 3, ants listed in Ferster & Prusak (1994); Ant Status; S, confirmed symbiont of MBB; pS, potential symbiont; u, unknown; P, potential predator.

	ENP	BHSP	Ant Status
Subfamily Pseudomyrmicinae			
Pseudomyrmex elongatus	1	2	P
Pseudomyrmex gracilis	1	-	P
Pseudomyrmex simplex	-	1	P
Subfamily Myrmicinae			
Crematogaster ashmeadi	-	1, 2	S
Monomorium floricola	1	2	u
Pheidole dentata	1	2	u
Pheidole floridana	1	1	u
Solenopsis invicta	-	1, 2	P
Solenopsis geminata	1	1, 2	u
Tetramorium simillimum	1	-	u
Wasmannia auropunctata	1	1, 2	P
Subfamily Dolichoderinae			
Forelius pruinosus	1	1, 2	S
Tapinoma melanocephalum	-	1	S
Subfamily Formicinae			
Brachymyrmex obscurior	1	1, 2	u
Camponotus floridanus	1, 3	1, 2	S
Camponotus planatus	1	1	S
Paratrechina bourbonica	-	1	pS
Paratrechina longicornis	1	1, 2	P

CHAPTER 8
CONCLUSIONS

This study successfully investigated the population genetics and corresponding conservation implications for the endangered Miami blue butterfly, *Cyclargus thomasi bethunebakeri*. Such knowledge is crucial for developing effective conservation and management strategies for this taxon and can help guide the decision-making process for other threatened invertebrate taxa.

I began this project by creating a microsatellite library to develop markers appropriate for addressing population-level questions. Developing microsatellites for butterflies can be difficult because of the high occurrence of null alleles (Zhang 2004), but microsatellites are the most appropriate markers for discerning gene flow and molecular variation within populations. Additionally, I utilized and tested a relatively noninvasive sampling technique that allowed me to sample living butterflies without negatively impacting them. Coupled together, the twelve polymorphic microsatellite markers and noninvasive sampling method allowed me to assess genetic diversity within populations of this imperiled taxon (Chapter 2).

I employed the use of microsatellite markers to explore historical genetic diversity by genotyping museum specimens of butterflies using single legs and labial palps (Chapter 3). Only one other study has successfully amplified microsatellite DNA from historical butterfly specimens, but this study destructively-sampled the heads (Harper et al. 2006). However, the Harper et al. (2006) study was able to amplify three microsatellite loci while I was only able to amplify one. My study of *C. t. bethunebakeri* from museum collections quantified the diversity present in a historical population present in Key Largo, Florida. According to the results from a single microsatellite locus, diversity was consistently high within the historical Key Largo population. My study then expanded beyond the genetic field to include an analysis of the

geographic distribution of this butterfly. Through examination of several museum collections and discussions with lepidopterists and collectors, I discovered that *C. t. bethunebakeri* were locally common in some areas of southern Florida and the Florida Keys up until the 1980s. During favorable years, small populations of butterflies were also established in more northern counties and they even colonized some inland areas, although none of these remained for more than a few seasons. The *C. t. bethunebakeri* population centers appear to be in southeast Florida and the Florida Keys. We can use the knowledge of historical distribution and population locations to plan future reintroduction events with captive-bred butterflies. We can also continue to search for additional wild populations of butterflies within areas that showed a high historical incidence of these butterflies.

The historical distribution of butterflies throughout the Florida Keys further suggests a metapopulation structure. This involved partially-isolated populations of *C. t. bethunebakeri* that were loosely connected via gene flow and that underwent localized extinction and colonization events along the chain of suitable habitat in the Florida Keys. Knowledge of historical levels of gene flow and population distribution will be useful in planning future reintroduction measures. While I could not assess historical gene flow between populations, I was able to confirm the presence and distribution of past populations by cataloguing museum specimens and through conversations with lepidopterists. The genetic diversity of the historical population in Key Largo was high, but diversity within this population was observed to decrease over the decades that I sampled (1940-1979). A comparison of historical diversity from the Key Largo population with the extant population on Bahia Honda State Park (BHSP) revealed differences in allelic frequencies, but only minor differences in the overall number of alleles. The historical population had a greater distribution of medium-high frequency alleles, while the BHSP

population showed dominance of a few alleles (Chapter 3). Again, this study was hindered by the presence of data from only one microsatellite locus, but this is an issue when dealing with museum materials and degraded DNA.

My investigations into the genetic diversity of the BHSP and Key West National Wildlife Refuge (KWNWR) populations revealed adequate levels of diversity within the 12 polymorphic microsatellite marker loci used to assess the populations. There was no evidence of a recent bottleneck within the BHSP population, but there may have been one in or prior to the 1980s that was undetectable with current methodologies. The KWNWR population did show signs of a bottleneck, which provides support for the hypothesis that it is a newly founded population. The BHSP population had higher levels of diversity than originally predicted from the low census estimates (Chapter 4). Gene flow between habitat patches on BHSP is crucial for the maintenance of this diversity and likely imperative for the long-term survival of the population. Future reintroduction efforts need to be mindful of this and mimic the metapopulation structure present on BHSP. There is gene flow between distant habitat patches on BHSP and the patches along the Main Road of BHSP appear to be especially important in maintaining this gene flow. These patches likely act as stepping stones and butterfly "rest stops" for the (relatively) long journey between the far ends of BHSP. It appears that a "mini-metapopulation" exists on Bahia Honda, one that is similar to the historical metapopulation structure and distribution of populations on a larger scale.

The captive colony has been extremely successful at producing individuals for reintroduction. However, genetic monitoring showed that the captive colony significantly diverged from the BHSP population after only a few generations with no new infusion of material from the wild (Chapter 5). Measures of genetic potential, such as allelic richness,

remained stable in the BHSP population but were observed to decrease over time in captivity. I have also recorded the appearance of novel alleles in the captive colony, which are of concern as they may represent variation that would be detrimental to individuals reintroduced to the wild. Individuals introduced from the wild will help prevent inbreeding depression in the captive colony and likely boost the fitness of organisms bred for reintroduction efforts (Tallmon et al. 2004).

Laboratory investigations into the effects of a small number of individuals founding new colonies did not go as expected, but did yield information which will be useful in other conservation endeavors. The discovery of a novel baculovirus highlights the vulnerability of captive-bred populations and has led to modified laboratory techniques. The importance of demographic factors like eclosion time, availability of suitable mates, etc. highlighted the importance of stochastic factors in colony founding and population persistence. The genetics of newly founded colonies were not able to be well-studied in these experiments due to small sample size, but the demographic factors highlighted how populations may be vulnerable before or while molecular issues are involved (Chapter 6).

The presence of symbiotic ant partners adds to the charismatic nature of the *C. t. bethunebakeri* story. My surveys showed that several ant genera and species were found tending *C. t. bethunebakeri* larvae and no predation by ants was directly observed. The presence of invasive ant taxa should be monitored or addressed by future study as these ants, while they might not directly prey on *C. t. bethunebakeri* larvae, may be responsible for disrupting the mosaic ant community which may eventually negatively affect immature *C. t. bethunebakeri* (Chapter 7).

Future Research

The markers I developed in this study will be useful for future conservation efforts with captive and wild populations of this butterfly and may even be applied to other members of the genus *Cyclargus*. If other members of the genus are imperiled in the Caribbean, these microsatellite markers may be useful in elucidating population structure and assessing genetic diversity.

The microsatellite markers were useful in discerning the genetic diversity present in the newly found population in KWNWR. This discovery on the remote, uninhabited islands of the refuge again speaks to the butterflies' ability to withstand difficult environmental conditions and lends hope for the recovery of the taxon. Despite extensive surveying efforts, I still believe it is likely that additional populations of butterflies exist in the Florida Keys, although these may be ephemeral and low in population number. The KWNWR population offers additional opportunities for study, not only on the biology of this organism but also on greater theoretical issues in conservation biology. Studies into host plant use and preference can be continued with this taxon as the larvae of the KWNWR population feed on a plant different from that on BHSP, an issue that may lead to ecological speciation or represent an ancestral polymorphism. Additionally, concerns of outbreeding depression are great within the conservation community, and experimental breeding between BHSP and KWNWR individuals may shed light on this topic. Conservation projects are often stuck in a dilemma between inbreeding depression and outbreeding depression (Edmands 2006) and the nature of these two populations coupled with the ability to raise this taxon in captivity may clarify this issue. From a management perspective, the KWNWR population also offers new genetic diversity, diversity that may prove essential in the long-term solution for returning fit individuals to the wild.

Recent theoretical models have been proposed to explain the concept of "genetic rescue" (Tallmon et al. 2006) and data from *C. t. bethunebakeri* may be useful in testing these models. Individuals from different metapopulation segments of BHSP may be capable of "genetically rescuing" other patches that have reduced variability. This may have been how the metapopulation structure of *C. t. bethunebakeri* worked historically, with immigrant butterflies "rescuing" neighboring population segments as well as founding new ones. Such hypotheses are supported by theoretical and experimental work with other metapopulations (Thrall et al. 1998; Ingvarsson 2001) and provide an interesting avenue for future research with *C. t. bethunebakeri*.

Concerns of genetic adaptation to captivity may be addressed by dividing the current captive colony into multiple captive colony segments. These colonies could be housed at additional institutions or maintained as discrete entities within the McGuire Center for Lepidoptera and Biodiversity. If a new virus or pathogen emerged and destroyed one colony, the taxon would be safeguarded by the presence of additional colonies in other locations. The continuous release of captive-bred individuals into the wild should be maintained in order to improve the chances of successfully founding new wild colonies. Additionally, newly-founded populations should be monitored regularly and definitive data about the success of larval vs. adult releases gathered and evaluated. A comparison of the successes of reintroductions from captive-bred individuals vs. translocated individuals from BHSP would also aid in reintroduction efforts. Given the expense of captive colonies and number of threatened taxa, such data could prove useful for other endangered invertebrate management projects.

Conservation Implications

The Xerces blue butterfly (*Glaucopsyche xerces*) was a lycaenid butterfly endemic to the coastal dunes of northern California. The development of the dune habitat undermined the remaining populations of these butterflies, and eventually led to its extinction in the 1940s. We

are in a time of mass extinction, with species such as the Xerces blue butterfly lost every day. Insect extinctions comprise the neglected majority of extinctions in our ongoing biodiversity crisis (Dunn 2005), and many insect and arthropod taxa are lost before they are even known to science. Recovery and conservation programs may be able to salvage some insect taxa, but the remaining diversity is lost forever.

While recovery programs such as that developed for *C. t. bethunebakeri* are noteworthy, it is more important that the factors leading to imperilment are identified and mitigated before species become endangered. Conservation endeavors should act more preemptively and aim to preserve species before they become threatened. This begins with the protection of habitat and a greater understanding of ecological networks and species' requirements. My work underscores the importance of knowledge of an organism's biology and ecology to its long-term persistence. These butterflies exist in a metapopulation framework for continued survival in the dynamic environment of south Florida, one that is dominated by major environmental changes precipitated by hurricanes and droughts. This butterfly has been able to exist in this system for a long time before people colonized the region, and its continued persistence in the region depends on a habitat network with colonies linked by gene flow between patches. Basic biological field work has uncovered the importance of habitat connectivity and the significance has been supported by my molecular studies.

The captive colony has been a productive endeavor in that we have learned useful biological information and have been successful at producing numerous organisms for reintroduction. However, the bureaucracy surrounding the reintroduction efforts and conflicting stakeholder interests may have hindered the initial success of the reintroduction goals. The development of the Miami Blue Working Group has helped mitigate some of these conflicts.

This group can serve as a model for other programs that aim to minimize discord between stakeholders and result in successful collaborations and reintroduction efforts (Daniels 2009).

A unified approach to conservation encompassing genetics, ecology, and behavior will offer a more comprehensive program with a higher chance of success, and the *C. t. bethunebakeri* recovery program has made great strides in encompassing all of these fields. As scientists we should further use the data we generate to make recommendations for practical solutions in management, especially where genetic data are concerned. Guidelines for the use of genetic data in both the listing process and management planning need to be drafted (Haig et al. 2006; Fallon 2007; Waples et al. 2007; Vernesi et al. 2008). Given the magnitude of the biodiversity crisis, there will only be more taxa listed in the future. New guidelines to assist in the effective use of genetic data will help standardize and streamline the listing process and aid in future decisions that will benefit the multitude of threatened organisms.

APPENDIX A
LIST OF ABBREVIATIONS

ABI: Applied Biosystems, Inc.
AMNH: American Museum of Natural History
A_R: Allelic richness
BHSP: Bahia Honda State Park, Florida
BNP: Biscayne National Park
bp: Basepairs
BSA: Bovine Serum Albumin
CC: Captive colony (of *Cyclargus thomasi bethunebakeri*)
CMNH: Carnegie Museum of Natural History
CNHM: Chicago Natural History Museum (the Field Museum)
DEP: Department of Environmental Protection
DNA: Deoxyribonucleic acid
D_S : Genetic distance
DJSP: Dagney Johnson Key Largo Hammocks Botanical State Park ENP: Everglades National Park
ESRI: Environmental Systems Research Institute (Redlands, California)
f: Inbreeding coefficient
FAM: Carboxyfluorescein (blue)
FLMNH: Florida Museum of Natural History
F_{IS}: The proportion of inbreeding in a population due to inbreeding within the subpopulation. This is the correlation between homologous alleles in an individual relative to the subpopulation.
F_{IT}: The proportion of inbreeding in a population due to inbreeding within the subpopulation and differentiation among subpopulations. This is the correlation between homologous alleles in an individual relative to the total population.
F_{ST}: The most inclusive measure of population substructure, it measures the proportion of inbreeding in the subpopulation relative to the total population (a level of genetic divergence among subpopulations). This value is used to estimate gene flow and population subdivision.
FWC: Florida Fish and Wildlife Conservation Commission, formerly abbreviated as FFWCC
GIS: Geographic Information System
GIS, Inc.: Genetic Identification Services, Inc. (Chatsworth, California)
H_O: Observed heterozygosity in a population based on the Hardy-Weinberg equilibrium principle.
H_E: Expected heterozygosity in a population based on the Hardy-Weinberg equilibrium principle.
HEX: Hexachlorofluorescein (green)
HWE: Hardy-Weinberg equilibrium. The equilibrium state of genotype frequencies achieved in an infinitely large, idealized population that is not subjected to mutation, migration, or selection. H_O and H_E values are used to test whether allelic frequencies conform to HWE.
IAM: Infinite alleles model; used to explain microsatellite mutation patterns.
N: Number of samples
N_A: Number of observed alleles
N_E: Effective population size.

n_e: Number of effective alleles
NPS: National Parks Service
P HWE: Probability that a population is in Hardy-Weinberg equilibrium. This value of often computed with a χ^2 test, but I use the exact test as recommended by Excoffier et al. (2005). The exact test extends the χ^2 test to a table of arbitrary size and uses a modified Markov chain walk to produce a more computationally efficient result (Guo & Thompson 1992).
PCR: Polymerase chain reaction
Rpm: Rotations per minute (of centrifuge).
R_{ST}: F_{ST} analogue for microsatellite markers (developed by Slatkin 1995)
SEM: Scanning electron microscope
SMM: Stepwise mutation model, used to explain microsatellite mutation patterns.
SMNH: Smithsonian Natural History Museum
T_a: Annealing temperature for PCR
T_m: Melting temperature
TET: Tetrachlorofluorescein (yellow)
TPM: Two-Phase Model; used to explain microsatellite mutation patterns.
USFWS: United States Fish and Wildlife Service
UV: Ultraviolet

APPENDIX B
DNA EXTRACTION METHODS

Day 1

Extraction from whole butterfly:

1) Measure the forewing from the wing tip to where the wing meets the body. Record this in the spreadsheet and the lab notebook.

2) Remove the wings and abdomen with a razorblade. Store the wings and abdomen in a labeled tube.

3) Slice open the thorax with the razorblade and expose tissue by continuing to cut the thorax into pieces, taking care not to lose any of the thorax pieces. Only process one specimen at a time and make sure not to cross-contaminate samples.

4) Put cut up thorax into a clean, labeled tube (use the 2mL tubes). Put the thorax into the tube by using the razorblade or forceps. Add 180 µL Buffer ATL. Macerate the tissue with a sterile blue pestle. Grind up the tissue as much as possible in the tube and keep as much of the tissue as possible in the tube.

5) Add 20 µL Proteinase K. Mix by vortexing for 30 seconds. If necessary, do a short centrifuge to make sure that all the tissue is in the liquid and is not stuck on the side of the tube or on the lid. Incubate at 55° C overnight. **PROCEED TO DAY 2**

Extraction from butterfly wing fragment:

1) Very carefully, remove the alcohol from the tube containing the wing fragment. I recommend centrifuging the sample for 2 minutes (at 13,200 rpm) to affix the wing fragment to the tube. Remove all alcohol by pipette.

2) Use the vacuum for 5 minutes to remove the rest of the alcohol.

3) Add 180 µL Buffer ATL and add 2 copper BBs (only use autoclaved BBs, never use small glass beads).

4) Vortex each sample for 30 seconds, ensuring that the BBs macerate the wing fragment. Tilt the tube around in the vortex to make sure that the BBs move all around the tube.

5) Very carefully, pour the BBs out of the tube into a waste cup. This is best done by pouring the BBs into the lid of the 2 mL tube and then dumping them out of the lid. You may want to practice pouring BBs out of a tube with water in it (and not a valuable sample). If it is very difficult to remove the BBs, switch to macerating wing tissue with the sterile blue pestles.

6) Add 20 µL Proteinase K. Centrifuge for a short spin (or for 1 minute if necessary) to make sure that all the fragment is in the liquid and not stuck on the sides or lid of the tube. Incubate at 55° C overnight. **PROCEED TO DAY 2**

Day 2

1) Remove the rack of tubes from the 55° C water bath. Turn the bath up to 70° C.

2) Add 20 µL (of 20 mg/mL concentration) RNAse to each sample. Let it sit for 5 minutes at room temperature. Mix by inverting tubes. Continue to Step 4 for wing fragment extractions. There is no need to centrifuge the tubes with wing fragments as there is not enough tissue to clog the spin columns.

3) Whole butterfly/thoracic extractions only: centrifuge the tube for 5 min. at 13,200 rpm. Transfer the supernatant to a new tube. Do this with a P1000 for most of the liquid and then get the remaining liquid with a P200 (yellow tip). Try to get as much liquid as possible into the new tube while minimizing the amount of tissue. Discard the tube with the tissue in it.

4) Add 200 µL Buffer AL to the sample and mix well by vortexing. Incubate at 70° C for 10 minutes. Flick or invert the tube a few times after removing it from the bath. Return the bath to 55° C.

5) Add 200 µL 100% ethanol and vortex for 15 seconds.

6) Label each spin column with the sample ID, but don't label the collection tubes. Pipet the mixture from step 4 into the DNeasy mini spin column placed in a 2 mL collection tube. Centrifuge at 8,000 rpm for 1 minute. Discard flow-through and collection tube.

7) Place the DNeasy mini spin column in a new 2mL collection tube (provided). Add 500 µL Buffer AW1 and centrifuge for 1 minute at 8,000 rpm. Discard flow-through and collection tube.

8) Place the DNeasy mini spin column in a new 2mL collection tube (provided). Add 500 µL Buffer AW2 and centrifuge for 3 minutes at 13,200 rpm. Discard flow-through and collection tube.

9) Carefully remove the column so that the column does not come into contact with the flow-through. If this does happen, empty the collection tube and reuse it in another centrifuge step for 1 min. at 13,200 rpm (rotations per minute).
10) During the 3 minutes that the samples are centrifuging, put the Buffer EB into the warm water bath.

11) Place the DNeasy mini spin column in a clean, labeled 2mL microcentrifuge tube (not provided). For whole body/thorax extractions: pipette 100 µL of warm Buffer EB (=10mM TRIS) directly onto the DNeasy membrane. For wing fragment extractions: pipette 50 µL of warm Buffer EB (=10mM TRIS) directly onto the DNeasy membrane.

Incubate at room temperature for 3-5 minutes. Centrifuge for 1 minute at 8,000 rpm to elute. Throw away the spin column and save the labeled tube in the -20℃ freezer.

APPENDIX C
ALLELE FREQUENCY TABLES

Table C-1. Allele frequency for locus CthB11.

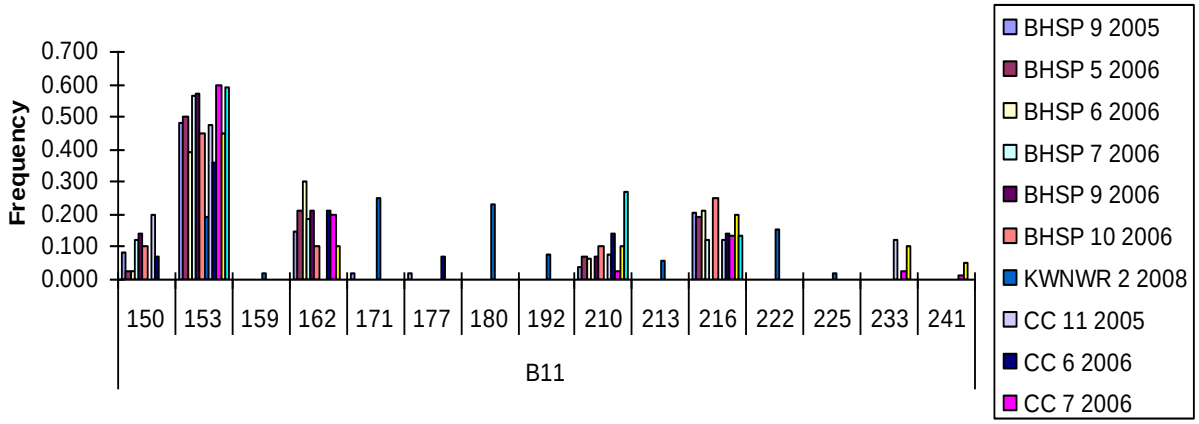


Table C-2. Allele frequency for locus CthB101.

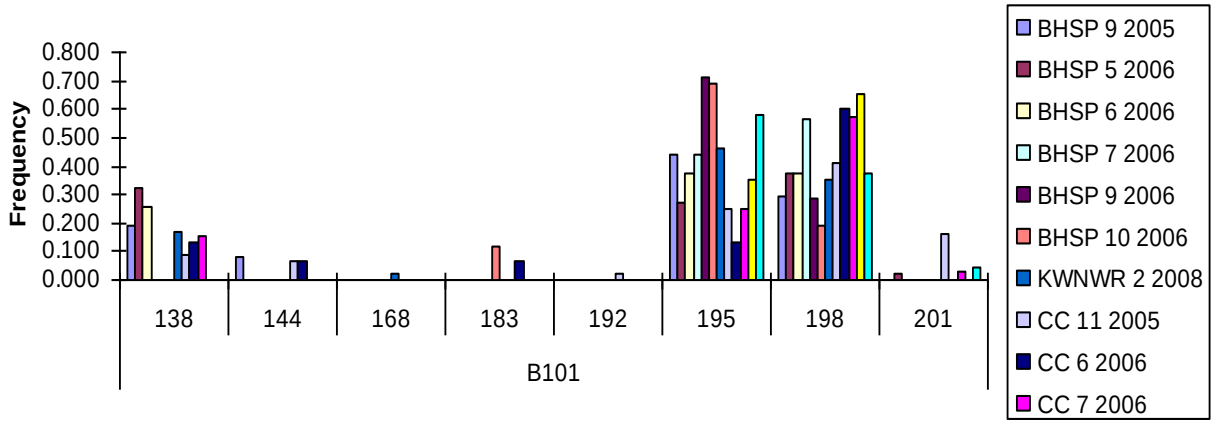


Table C-3. Allele frequency for locus CthB103.

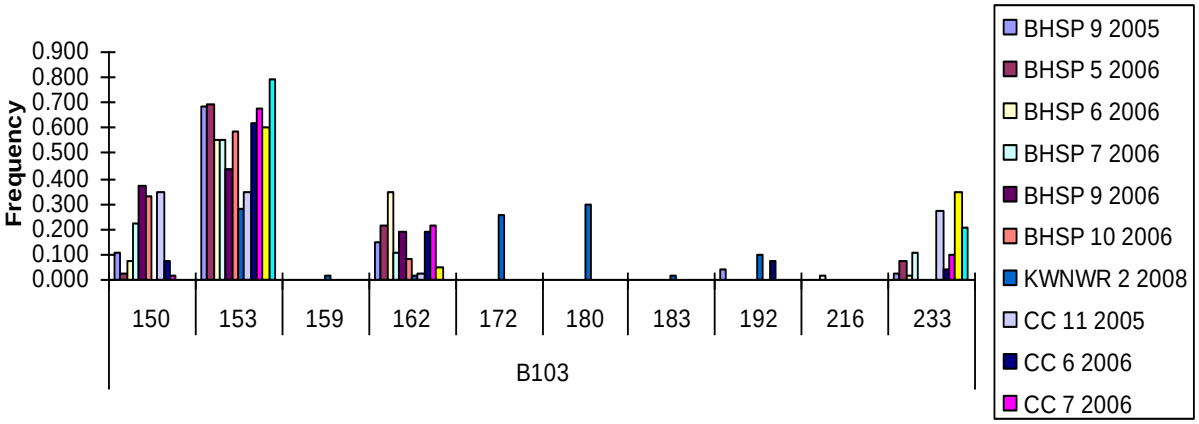


Table C-4. Allele frequency for locus CthB106.

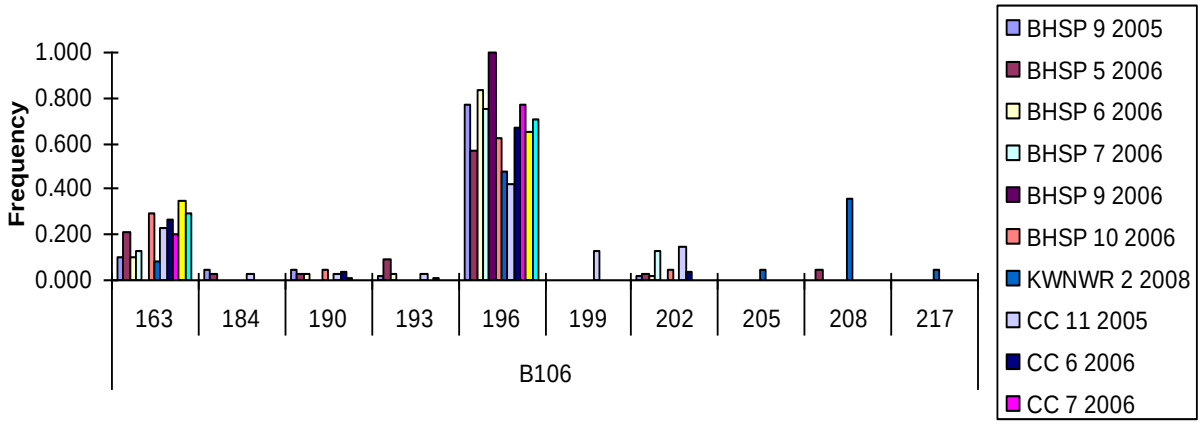


Table C-5. Allele frequency for locus CthB115.

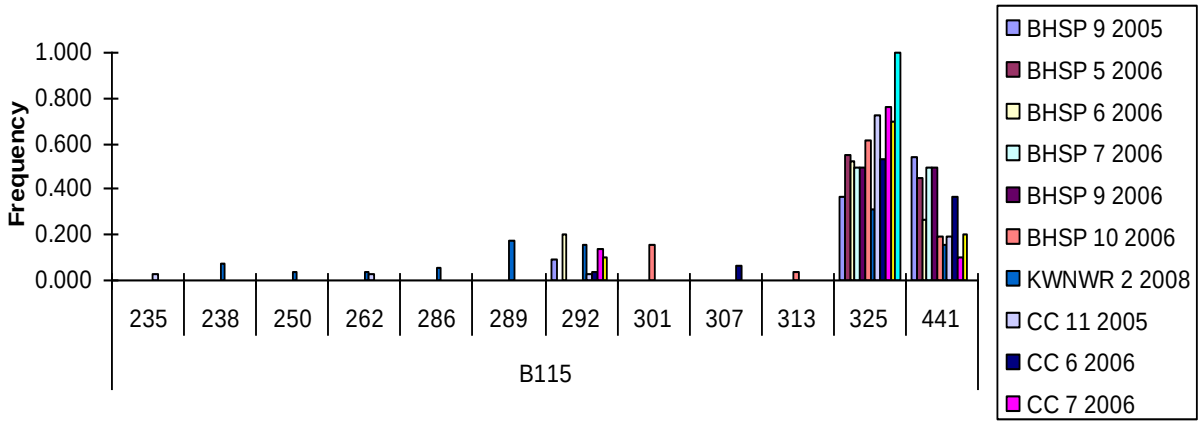


Table C-6. Allele frequency for locus CthB117.

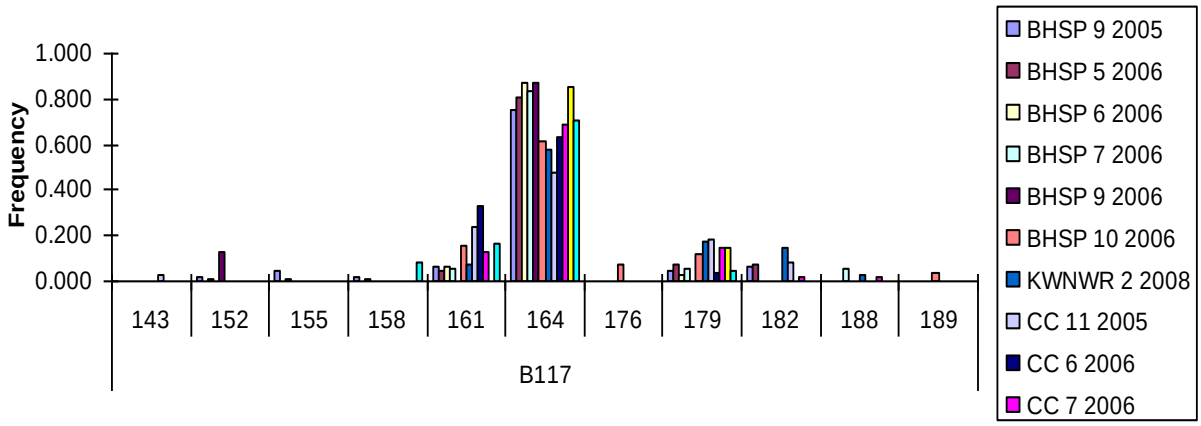


Table C-7. Allele frequency for locus CthB119.

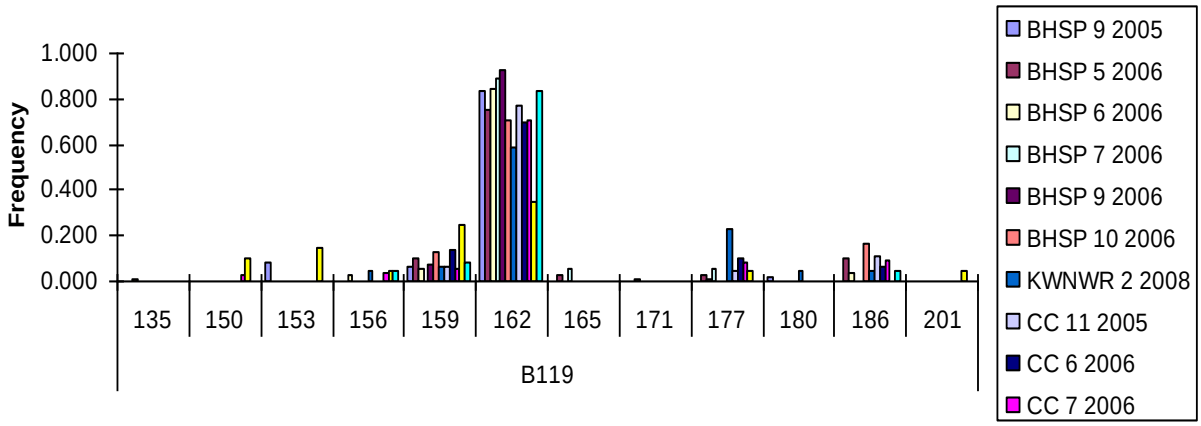


Table C-8. Allele frequency for locus CthC12.

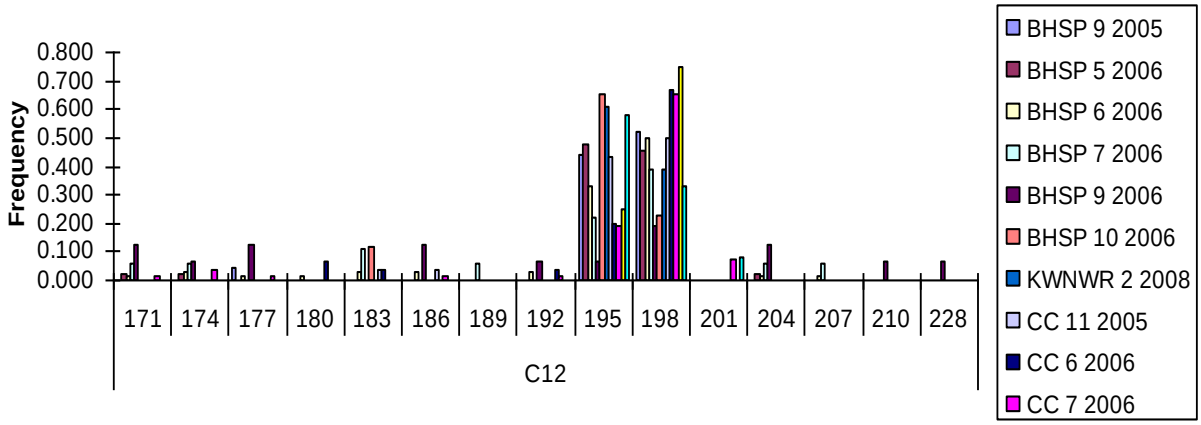


Table C-9. Allele frequency for locus CthC116.

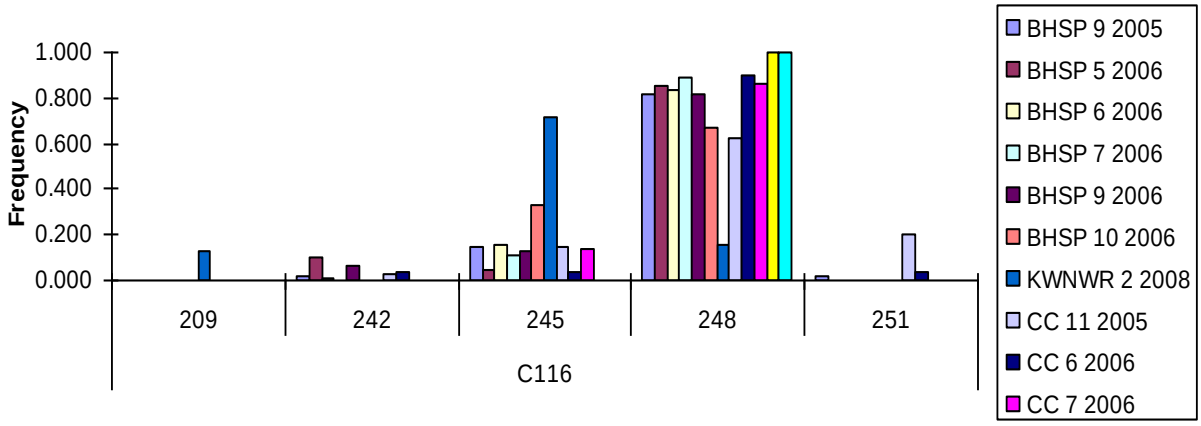


Table C-10. Allele frequency for locus CthC124.

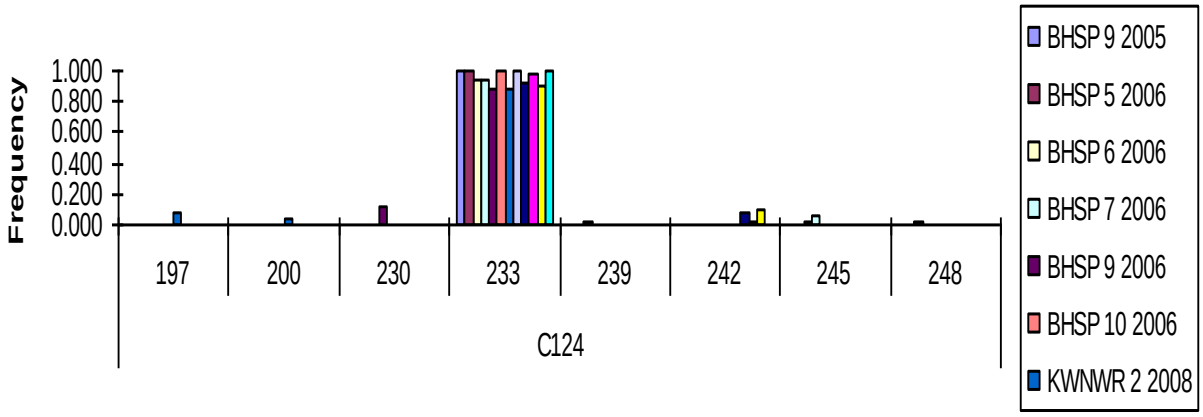


Table C-11. Allele frequency for locus CthC127.

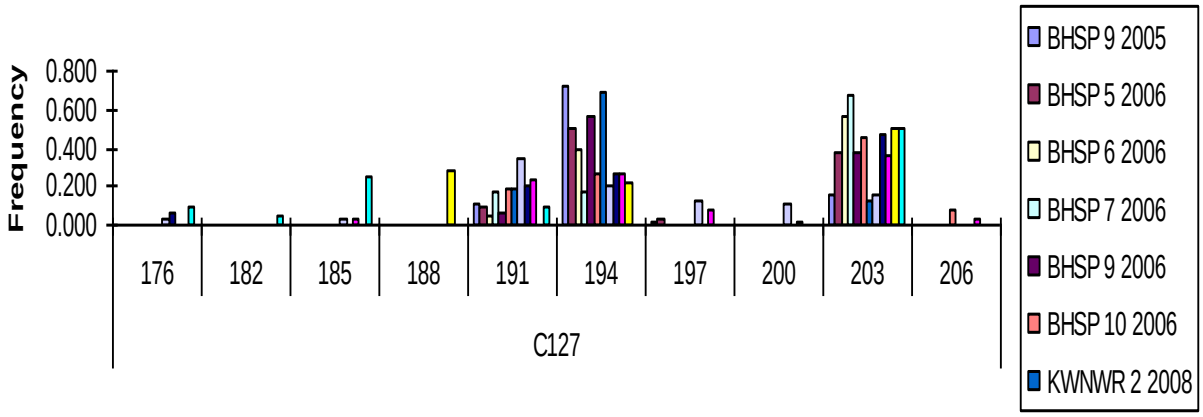
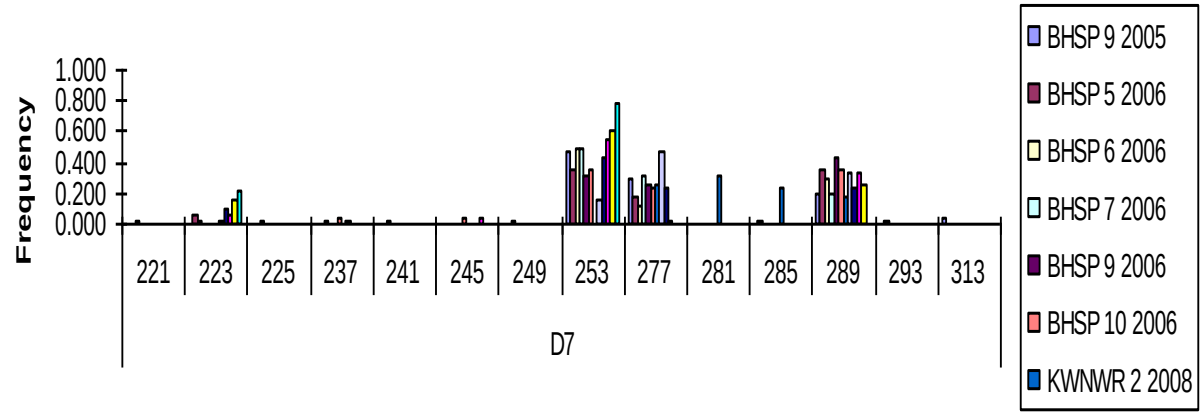


Table C-12. Allele frequency for locus CthD7.



APPENDIX D
PRIVATE ALLELE LIST

Table D-1. Private allele list.

Population	Locus	Allele (bp)	Frequency
BHSP 2005-2006	B11	171	0.005
BHSP 2005-2006	B103	216	0.004
BHSP 2005-2006	B106	208	0.009
BHSP 2005-2006	B115	301	0.018
BHSP 2005-2006	B115	313	0.005
BHSP 2005-2006	B117	152	0.018
BHSP 2005-2006	B117	155	0.013
BHSP 2005-2006	B117	176	0.009
BHSP 2005-2006	B117	189	0.004
BHSP 2005-2006	B119	135	0.005
BHSP 2005-2006	B119	165	0.009
BHSP 2005-2006	B119	171	0.005
BHSP 2005-2006	B119	180	0.005
BHSP 2005-2006	C12	189	0.004
BHSP 2005-2006	C12	204	0.022
BHSP 2005-2006	C12	207	0.009
BHSP 2005-2006	C12	210	0.004
BHSP 2005-2006	C12	228	0.004
BHSP 2005-2006	C124	230	0.009
BHSP 2005-2006	C124	239	0.004
BHSP 2005-2006	C124	245	0.009
BHSP 2005-2006	C124	248	0.009
BHSP 2005-2006	D7	221	0.005
BHSP 2005-2006	D7	225	0.009
BHSP 2005-2006	D7	241	0.005
BHSP 2005-2006	D7	249	0.009
BHSP 2005-2006	D7	285	0.005
BHSP 2005-2006	D7	293	0.005
BHSP 2005-2006	D7	313	0.009
CC 2005-2006	B11	233	0.067
CC 2005-2006	B11	241	0.010
CC 2005-2006	B101	192	0.005
CC 2005-2006	B106	199	0.025
CC 2005-2006	B115	235	0.005
CC 2005-2006	B115	262	0.005
CC 2005-2006	B115	307	0.010
CC 2005-2006	B117	143	0.005
CC 2005-2006	B119	150	0.020
CC 2005-2006	B119	201	0.005
CC 2005-2006	C12	201	0.043
CC 2005-2006	C124	242	0.031
CC 2005-2006	C127	176	0.027

Table D-1. Continued

Population	Locus	Allele (bp)	Frequency
CC 2005-2006	C127	182	0.005
CC 2005-2006	C127	185	0.043
CC 2005-2006	C127	188	0.022
CC 2005-2006	C127	200	0.027

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BIOGRAPHICAL SKETCH

Emily Virginia Saarinen was born in upstate New York. She completed school in the Fayetteville-Manlius public school district and attended Syracuse University as a linguistics and anthropology major in the Honor's Program from 1995-1997. As her interests in the biological sciences began to grow, she transferred to New College, at that time the Honor's College of the University of South Florida. It was in Sarasota, Florida at New College that her interests in entomology and the natural sciences really blossomed. Under the expert tutelage of Drs. Lee and Jackie Miller and Sandra Gilchrist, Emily embarked on a study of the coevoution between a local moth species and its host plant. She graduated from New College in 1999 with degree in biology and a desire to explore the natural world.

Emily spent the next 12 months travelling around the world, visiting Denmark, Oman, Sri Lanka, Indonesia, Thailand, and Malaysia. She returned to the U.S. with a desire to pursue a degree in entomology after her experiences overseas. After working in industry for a year, she began her M.S. degree at the University of Florida in the Department of Entomology and Nematology. She was awarded a Fulbright Fellowship from the U.S. State Department and conducted field and laboratory research at the Forest Research Institute of Malaysia in 2002-2003. Her research on the symbiotic relationship between highly specialized weaver ants and lycaenid butterflies in the rainforest of Malaysia solidified her desire to obtain a doctorate in entomology. She finished her M.S. degree in 2004 and quickly began her Ph.D. research on the Miami blue butterfly, focusing on the interface between ecology and genetics. She received her Ph.D. from the University of Florida in the spring of 2009.

Emily continues to explore the natural world and is joined by her husband and daughter. Emily's plans are to continue teaching and conducting research around the world and to apply her knowledge to conservation projects.