Final Report

Research to Support Captive Rearing and Release of Palos Verdes Blue Butterfly

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The Urban Wildlands Group, P.O. Box 24020, Los Angeles, California 90024-0020

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Introduction

Glaucopsyche lygdamus palosverdesensis Perkins and Emmel (Palos Verdes blue butterfly) was described as a subspecies endemic to the Palos Verdes Peninsula in southwestern Los Angeles County. It is one of 10 subspecies of *Glaucopsyche*, which are distributed across most of North America and into eastern Siberia (Mattoni 1995). The Palos Verdes blue butterfly was listed as an endangered species in 1980 (45 Federal Register 44939), and by 1983 was considered to be extinct (Arnold 1987, Arnold 1990, Mattoni 1993). It was subsequently discovered at a previously unknown location in 1994 (Mattoni 1995), presenting an unparalleled opportunity for recovery of an "extinct" species.

Immediately after discovery of a population of Palos Verdes blue butterfly at the Defense Fuel Support Point (DFSP) in San Pedro, California, a captive breeding program was initiated to guard against extinction while habitat restoration was undertaken (Mattoni et al. 2003). This captive breeding program has continued through today, with periodic input of wild individuals to enhance the genetic diversity of the captive population (Johnson et al. 2008, Mattoni and George 2002, Mattoni et al. 2005).

Nothing is known of the genetic diversity of either the captive population of Palos Verdes blue butterfly, nor of the extant or historic wild populations. Knowledge about such variation is important to conservation actions, especially reintroduction and captive rearing, for several reasons. First, the source population for the captive breeding program has always been small, with estimates ranging from roughly 30 to under 400 adults flying during any given year from 1994 to 2007 (Longcore 2008), and could have already gone through a genetic bottleneck. Second, the limited input of wild genetic material to the captive population could have resulted in decreased diversity in the captive population, or drift within that population. Third, the physiography of the DFSP site is different from the other known historic locations for the species. DFSP is north-facing with less fog than the south-facing mid-elevation sites on the seaward side of the Palos Verdes Peninsula (Figure 1). As a result, the DFSP population may differ genetically from the populations from which the subspecies was described. Knowledge of each of these genetic elements would aid captive breeding and reintroduction efforts.

Given the need to describe DNA with limited tissue, most current research on the genetic structure of populations of endangered butterflies uses microsatellites (Zeisset et al. 2005). Libraries of polymorphic microsatellite loci have been developed for a number of lycaenid butterflies that are of conservation concern, including Miami blue butterfly (Saarinen et al. 2009), two large blue butterflies (Zeisset et al. 2005), violet copper (Habel et al. 2008), and Karner blue butterfly (Anthony et al. 2001). For this project we have undertaken the creation of a microsatellite library for the Palos Verdes blue butterfly using specimens from the captive population to find and describe polymorphic microsatellite markers for the purpose of population and conservation studies.

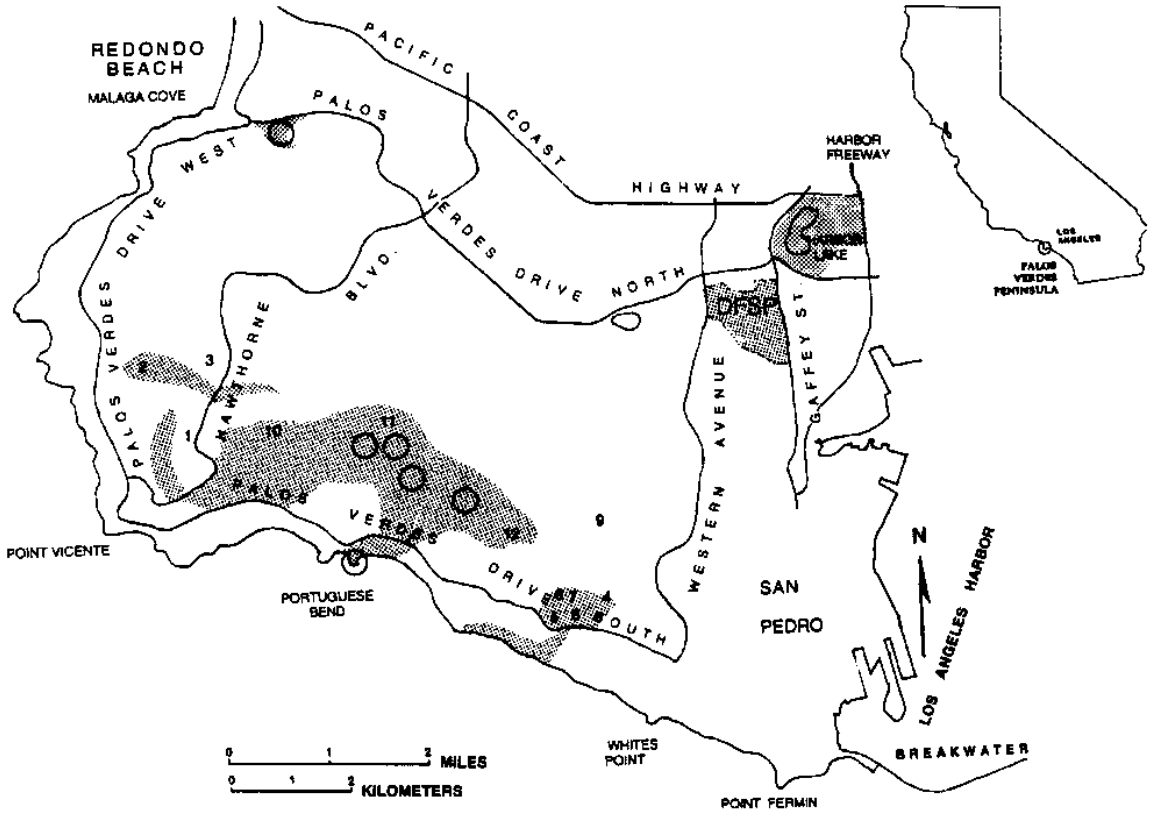

Figure 1. Map of the known distribution of Palos Verdes blue butterfly before its disappearance in 1984 (Mattoni 1995). 1. Hesse Park, 2. Agua Amarga Canyon, 3. Alta Vista Terrace, 4–8. Palos Verdes Drive East ("The Switchbacks"), 9. San Pedro Hill, 10. Upper Filiorum, 11. Crenshaw extension, 12. Klondike Canyon.

Methods

We enriched a genomic library of *Glaucopsyche lygdamus palosverdesensis* for trinucleotide microsatellites based on the protocol of Bardeleben et al. (2004). We selected three microsatellite motifs used in previous studies of butterflies – AAT, ATG and CTT (Van't Hof et al. 2005).

Twenty-five dead individuals were recovered from the captive colony after the 2008 flight season. These were the offspring of captive individuals, with no wild input to the colony for 5 years. For these specimens genomic DNA (gDNA) was extracted from the abdomen and thorax of each individual using a QiaAmp DNA Mini-Kit (QIAGEN). We pooled DNA from four individuals and digested 10 µg separately with Sau3A1 or HaeIII enzymes (New England Biolabs). Oligo A and B adaptors (Bloor et al. 2001) were generated and ligated to gDNA. Each ligated gDNA product was size-selected on a 1.5% agarose/TAE gel. DNA in the 0.5–1.5 kb range was excised from the gel and purified by a MinElute Gel Kit (Qiagen). All hybridizations were carried out using ~1 µg of DNA with 50 nm biotin-labelled probe [5′-

(Motif)$_7$GTGA(Biotinyl-T)C-3′], as previously described (Bardeleben et al. 2004). Enriched DNA was eluted from the magnetic beads using water preheated to 99°C, then amplified using polymerase chain reaction (PCR) with Oligo A as a primer. A second round of hybridization was carried out using 25 µL of this PCR product and identical conditions to the first round selection. Enriched gDNA was cloned using a TOPO TA pCR 2.1 Cloning Kit (Invitrogen), following the manufacturer's protocol. A total of 307 white colonies were picked with 99 possibly containing repeat regions. These were then sequenced using an Applied Biosystems (ABI) BigDye Terminator version 3.1 Cycle Sequencing Kit, and the M13 (–20) forward primer.

PCR products were sequenced on an ABI 3730XL sequencer. Thirty-nine sequences were unique and contained tri-nucleotide repeats. For thirty-one sequences that contained sufficient flanking sequence, primers were designed using Primer 3 (frodo.wi.mit.edu). An M13-hybrid primer process was used, and thus, the M13F (–20) sequence was added to the 5′ end of each forward primer (Boutin-Ganache et al. 2001). The thirty-one primer sets were first tested on a panel of 8 individuals for amplification and readability evaluation. The PCR mixtures contained the following reagents in a 10 µL volume: ~45 ng of DNA, 2× Multiplex Mix (QIAGEN), 0.4 mg/mL BSA, 0.1 µm forward primer, 0.1 µm 6-FAM dye-labeled M13 primer (ABI), and 2 µm reverse primer. The following step-down PCR cycle was used initially for each locus: 95 °C for 15 min, 25 cycles of 94 °C for 30 s, 59 °C for 1.5 min, and 72 °C for 1 minute, followed by 20 cycles of 94 °C for 30 s, 53 °C for 1.5 min, and 72 °C for 1 min, with a final extension of 60 °C for 30 min. PCR amplifications were performed on a Mastercycler ep (Eppendorf). Amplified PCR products were mixed with LIZ500 size standard, characterized on a 3730XL sequencer, and scored using GeneMapper 4.0 (all ABI).

Based on amplification result quality, thirteen trinucleotide microsatellite markers were tested on the full panel of 25 individuals. GENEPOP version 3.4 was used to calculate: 1) whether each marker was in Hardy Weinberg equilibrium (HWE), and 2) linkage disequilibrium (Raymond and Rousset 1995). We used a Bonferroni correction (Rice 1989) for multiple comparisons. CERVUS version 3.0 (Kalinowski et al. 2007) was used to determine the expected and observed heterozygosity for each marker.

Results

Nine markers were polymorphic and able to be typed in >90% of individuals and their characteristics are presented in Table 1. One marker was monomorphic for the set of 25 individuals tested and three markers that were polymorphic proved problematic in amplification and were not able to be amplified in the minimum number of individuals to be usable at this time (UCLA is working to optimize primer designs and amplification conditions for these markers so that they may ultimately be added to the microsatellite panel). The number of alleles per marker ranged from 3 to 11, with an average number of alleles per locus of 6.11. No markers were found to be in linkage disequilbrium and only markers Glp_029 and Glp_120 were found to be out of HWE (both had a heterozygosity deficit). The overall observed heterozygosity (H_O) for the 9 markers is 0.462 and the expected heterozygosity (H_E) is 0.600.

Table 1. Characterization of 9 novel loci tested in *Glaucopsyche lygdamus palosverdesensis*.

Locus	Primer sequences (5'-3')	Repeat motif	Product Size Range (bp)	N_A	H_O	H_E
Glp_029	F: TGTAGACCAGGGGAAGAACATC R: ATGCGAAGGTGAGAGTGGC	$(ATG)_4GCGGTG(ATG)_3$	326–347	4	0.292	0.515
Glp_038	F: GGCACGGTTCTCTATCAAGC R: TCTCGCATCACACTCATTGC	$(ATG)_{12}$	151–256	7	0.458	0.613
Glp_044	F: CCAGTATTCGTAACCATTGCC R: GCCATCTTGTTTGGGTGAG	$(ATG)_6$	233–245	4	0.333	0.487
Glp_120	F: CCATCATCAGGTTTTCACC R: GTGGTTTTGGATTTGTCTG	$(ATG)_2(GTG)_2(ATG)_6$	310–386	11	0.455	0.876
Glp_40	F: GCTTCCTGCTGCTTGTTTATG R: CCACTGATACTGCCACCACC	$(ATG)_{13}$	312–342	3	0.294	0.358
Glp_148	F: AGCAGTGGACGGCGATAG R: TGTGCGCAGTACACAAGAGC	$(ATG)_8$	214–272	4	0.435	0.583
Glp_153	F: TGCGCTTTTATTGGTCACG R: CCCAAGTTGGTGTTCAACG	$(ATG)_6ATC(ATG)_1$	190–211	4	0.440	0.401
Glp_161	F: ATGGTTAGCGCTCATTGGAG R: GCCATCTGAAAAGGGTTGAC	$(ATG)_{12}TTG(ATG)_{10}$	147–180	10	0.800	0.849
Glp_172	F: ACCGATACACAACGATACGC R: CATGTGAGCTGGAAAACAAATATC	$(ATG)_{12}$	97–213	8	0.652	0.720

N_A, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity. Note that the M13 (-20) forward sequence was added to the 5′ end of each forward primer.

Discussion

The microsatellite library of 9 markers for *Glaucopsyche lygdamus palosverdesensis* has suitable characteristics for subsequent use in population and conservation genetics studies. The 9 markers are well behaved for the evaluation set of 25 individuals, with no markers in linkage disequilibrium and only two markers out of HWE. The higher overall expected heterozygosity (H_E) value of 0.600 compared to the overall observed heterozygosity (H_O) value of 0.462 indicates a homozygote excess, which is due to the small population size and probable genetic bottleneck that has occurred in this species both in the wild population and the captive colony.

As a comparison with other recent butterfly microsatellite libraries, the microsatellite library characteristics of another endangered butterfly with a small population size, the Miami blue butterfly (Saarinen et al. 2009), show that 7 of 12 markers were out of HWE (heterozygote deficit). In our library, 2 of 9 markers are out of HWE (heterozygote deficit). In the Miami Blue study of 114 genotyped individuals, they observed an average number of alleles per locus of 7.75. Considering the difference in sample size (114 vs. 25 for our study), the average num-

ber of alleles observed for the Palos Verdes blue butterfly of 6.11 is comparable. We do observe slightly higher H_O and H_E for the Palos Verdes blue sample set compared to the Miami blue sample set (H_O of 0.462 vs. 0.387 and H_E of 0.600 vs. 0.495). Further comparison with other recent butterfly microsatellite libraries (Anthony et al. 2001, Van't Hof et al. 2005, Zeisset et al. 2005) also indicates that this microsatellite library has comparable properties, and will allow multiple questions pertaining to genetic diversity to be answered.

The characteristics of the Palos Verdes blue butterfly microsatellite library we have developed looks to have the required resolving power and is suitable for subsequent population and conservation studies of the wild and captive colony populations. Funding from the current contract was sufficient only to develop the microsatellite library. The collaborators will continue with genetic analysis with other funding sources. With additional funding we will characterize the diversity of a greater number of specimens from the captive colony and wild population.

Future efforts will be concentrated on attempts to characterize the historical levels of genetic diversity in the wild populations. Acquiring museum specimens (Harper et al. 2006) to genetically estimate historic population sizes, genetic diversity (e.g. heterozygosity) and other historical demographics (e.g. signatures of population increase, decline) will provide the critical information for efforts towards release of individuals.

All expenditures on this contract were used to acquire materials and for labor in the laboratory. All salaries for senior personnel at The Urban Wildlands Group, Moorpark College, and UCLA were reallocated to project labor and supplies and participation of these individuals was made as in-kind donations. In addition, UCLA provided matching support for laboratory expenses.

This research was conducted under the authority of the United States Fish and Wildlife Service's (USFWS) Biological Opinion on the Formal Section 7 Consultation for the Chevron 1 8" Pipeline and Associated Government Pipelines Project, Defense Fuel Support Point, San Pedro, Los Angeles County, California (1-6-96-F-09).

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