8-1-2008

Population Structure of Limenitis Butterflies in Hickman, Kentucky

Mollie Johnson
Western Kentucky University

POPULATION STRUCTURE OF *LIMENITIS* BUTTERFLIES IN HICKMAN, KENTUCKY

A Thesis
Presented to
The Faculty of the Department of Biology
Western Kentucky University
Bowling Green, Kentucky

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science in Biology

By
Mollie R. Johnson

August 2008

POPULATION STRUCTURE OF *LIMENITIS* BUTTERFLIES IN HICKMAN, KENTUCKY

Date Recommended 6/20/08

Jeffrey D. Harris
Director of Thesis

Cheryl O. Davis

Claire A. Rinehart

Richard G. Bowker 9/3/2008
Dean, Graduate Studies and Research Date

DEDICATION

This manuscript is dedicated to Sara Ann Neff and Amber Jane Johnson.

ACKNOWLEDGEMENTS

First and foremost I would like to thank my family for all of their love, support, motivational speeches, and of course, financial assistance throughout the years. My mom and dad have been a constant source of inspiration for me. They let me know at a very young age that there were no limits to the things that I could do with my life. Ballerina, doctor, firefighter, actress, concert pianist, or the President of the United States...the sky was the limit! I am one lucky girl to have been blessed with such an amazing mom and dad. My sister, Carrie, is responsible for helping me to keep my sanity over the years. She was always there for me when I needed a late-night pep talk or a motivational kick in the pants! My brother, Zachary, always reminds me to take things one day at a time. Zach is the type of person who knows how to enjoy the small things that life has to offer and keeps me grounded. He also gives the best bear hugs! Again, I got lucky in the brother and sister department. The rest of my family also deserves a tremendous thank you for all of their love and support throughout all of my educational endeavors. Without such an amazing support system I would not be the person that I am today.

Next, I would like to thank Dr. Jeffrey Marcus for teaching me how to be a biologist. I learned more about biology in the two years that I spent in his lab than I did in all of my previous years of schooling. Mostly, I would like to thank him for putting up with me, my loud mouth, and my tendency to be high-strung. Dr. Marcus gave me the tools and the inspiration necessary to achieve my goals and for that I am truly grateful. I would also like to thank all of the members of the Marcus lab: Tia Hughes, Tim Shehan, Brooke Polen-Jackson, Tara Powell-Cox, Rachel Barber, Michelle Dodson, Aaron Edwards, Jauan Burbage, Alan Simmons, and Joey Marquardt. Tia, Tim, and Rachel

spent a lot of long days in the field catching butterflies, shopping for rotten fruit, blending up butterfly bait, wearing butterfly bait, taking a bath in insect repellant, and picking unwanted ticks off of their clothes and skin. They have all been very helpful and supportive and made my graduate school experience truly amazing. I could not have asked for a better group of people to share this chapter in my life with.

Finally, I would like to thank several individuals for their generous contributions and assistance with this project. Dr. Cheryl Davis and Dr. Claire Rinehart were on my graduate committee and provided encouragement and guidance throughout my time at WKU. Dr. John Andersland, Dr. Charles Covell, and Dr. Jeffrey Marcus were kind enough to allow me to use their amazing photographs in this manuscript. Jenny Stovall and John Sorrell also deserve a big thank-you for helping me in the Biotechnology Center on numerous occasions.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

POPULATION STRUCTURE OF *LIMENITIS* BUTTERFLIES IN HICKMAN, KENTUCKY

Mollie R. Johnson August 2008 Pages: 49

Directed by: Jeffrey Marcus, Cheryl Davis, and Claire Rinehart

Department of Biology Western Kentucky University

Two species of *Limenitis* butterflies occur along the Mississippi River at Hickman, Kentucky: the viceroy, *L. archippus*, and the red-spotted purple, *L. arthemis astyanax*. *Limenitis archippus* occurs at a frequency that is 10-30 times more abundant than its congener, *L. arthemis astyanax*. Interspecific matings between *L. archippus* and *L. arthemis astyanax* are very rare in the wild and give rise to a hybrid form *Limenitis archippus* X *L. arthemis astyanax* form *rubidus*. Only 7 heterospecific pairings between the parental species and 72 "*rubidus*" individuals have been documented in the wild in all of North America. Of these documented cases, 2 heterospecific mating pairs and 2 *rubidus* individuals have been collected along a single 100 meter stretch of the Mississippi River at Hickman over the last several years, suggesting that this may be a "hybridization hotspot".

Molecular analysis of mitochondrial DNA haplotype, nuclear SNPs and nuclear Randomly Amplified DNA Fingerprints (RAF) from the 2 Hickman *rubidus* butterflies confirms that they are both F_1 hybrids and that *L. archippus* was the maternal parent for each specimen. I am interested in discovering the extent to which hybridization has allowed gene flow between *L. archippus* and *L. arthemis astyanax* at this location.

Ten individuals of each species were collected at Hickman and analyzed for both mitochondrial DNA haplotype and nuclear Randomly Amplified DNA Fingerprint (RAF) markers. The results of my analyses suggest that there may be some ongoing gene flow between these two species of *Limenitis* at this site.

INTRODUCTION

Two species of *Limenitis* butterflies occur along the Mississippi River at Hickman (Fulton County), Kentucky; the viceroy, *Limenitis archippus* and the red-spotted purple, *Limenitis arthemis astyanax* (Covell 1994). These two species of butterflies are very closely related and were once thought to be sister species (Willmott 2003). Sister species are species that share a most recent common ancestor. Even though they are very closely related, *L. archippus* and *L. arthemis astyanax* are dissimilar in color pattern because they mimic different distasteful species.

The genus *Limenitis* includes several species that are well-known mimics (Poulton 1909). The viceroy (*L. archippus*), along with the monarch (*Danaus plexippus*), makes up one of the earliest documented and most widely appreciated examples of a mimicry complex in nature (Poulton 1909; Walsh & Riley 1869). The viceroy butterfly, *L. archippus*, is dark orange with black markings along its veins and has a row of white spots that border the edge of the wings (Figure 1). The color and pattern of *L. archippus* closely mimics the distasteful monarch butterfly, *D. plexippus*, with the exception of a horizontal black stripe that crosses the bottom of its back wings (Figure 1) (Platt 1983). The caterpillars of *D. plexippus* feed on milkweed (genus *Asclepias*), which is a toxic plant (Brower et al. 1968). *Asclepias* produces cardiac glycosides which serve as a chemical defense against predators (Brower et al. 1968). *D. plexippus* caterpillars can tolerate the cardiac glycosides and therefore, are able to feed on this plant unharmed (Brower et al. 1967). As a result, *D. plexippus* has little competition for this foodplant. *Danaus plexippus* feeds on the leaves of the milkweed and stores the poisonous

glycosides in its body (Brower et al. 1967). The adult form of *D. plexippus* also retains the toxins, which makes them distasteful to predators (Brower et al. 1968). Despite their distastefulness, *D. plexippus* has a bright and obvious coloration that is easily observed by visual predators, making D. *plexippus* an easy target. When a predator, such as a bird, eats *D. plexippus* the poisonous glycosides cause the bird to vomit (Brower et al. 1968). This system is beneficial to *D. plexippus* because the birds learn from experience to equate the color pattern of *D. plexippus* with a bad taste (Brower et al. 1968). As a result, many predators avoid consuming *D. plexippus.*

Limenitis archippus and *D. plexippus* were once thought to be a classic example of Batesian mimicry (Vane-Wright 1991). Batesian mimicry involves a palatable species mimicking an unpalatable model (Ritland & Brower 1991). Studies conducted by Jane Van Zandt Brower in the 1950's show that the situation is somewhat more complicated. The results of Brower's study indicated that *L. archippus* is more palatable than *D. plexippus*, but less palatable than the non-mimetic butterflies used in the study (Brower 1958a). The studies conducted by Jane Van Zandt Brower showed that L. *archippus* is somewhat distasteful (Brower 1958a). The results of this study were interpreted by Brower and the scientific community as support for the Batesian mimicry hypothesis (Brower 1958a).

More recent studies conducted in the early 1990's by David Ritland and Lincoln Brower reassessed the widely accepted theory that *L. archippus* and *D. plexippus* are classic examples of Batesian mimicry (Ritland & Brower 1991). Ritland and Brower used red-winged blackbirds in their bioassay and their results suggest that *L. archippus* and *D. plexippus* are both very unpalatable (Ritland & Brower 1991). The theory of

Müllerian mimicry holds that two unpalatable species resemble each other and are both protected from predation as a result of their resemblance (Sheppard & Turner 1977). According to the definition of Müllerian mimicry and the results of Ritland and Brower's bioassay, this classic example of Batesian mimicry may not be an example of pure Batesian mimicry, but rather may include strong elements of Müllerian mimicry (Platt & Greenfield 1971; Ritland 1998; Ritland & Brower 1991). In Florida, where the monarch is less common than its congener, the queen (*D. gilippus berenice*), the Florida subspecies of the viceroy (*L. archippus floridensis*) appears to be a Müllerian mimic of the queen (Ritland 1991; Ritland 1998).

What is less widely appreciated is that there are additional mimics in the genus *Limenitis*. The red-spotted purple, *L. arthemis astyanax*, is thought to mimic the pipevine swallowtail, *Battus philenor* (Figure 1) in the southeastern United States (Brower & Brower 1962; Platt & Brower 1968). *L. arthemis astyanax* is black and has iridescent blue and purple dorsal wings, while the ventral wings showcase the red-orange marginal spots that gave this butterfly its common name (Platt 1975). Eyespots are generally located away from the butterfly's head and deter predators away from the body and vital organs of the butterfly and attract their attention to the wings instead (Stevens 2005). This can allow the butterfly to escape with only a hole in its wing, which does not compromise its ability to fly (Hill & Vaca 2004). Some species of butterfly are able to fly with as much as 70 percent of their wings missing (Morgan & Marent 2008). The caterpillars of *B. philenor* feed on Dutchman's Pipe, *Aristolochia macrophyilla*, and other species of *Aristolochia* (Triplehorn & Johnson 2005). *Aristolochia* produces the alkaloid aristolochic acid which makes the plant highly toxic. *Battus philenor* caterpillars feed on

Aristolochia and their body retains the toxins (Ramos 2001). Adult *B. philenor* are unpalatable to predators and as a result, predators will avoid eating these butterflies (Brower 1958b). *Limenitis arthemis astyanax* is a palatable species that has evolved to mimic *B. philenor,* (Brower & Brower 1962; Platt & Greenfield 1971) suggesting that it is a Batesian mimic.

The similarly colored *L. arthemis arizonensis*, which lives in the Southwestern United States and Mexico, is also thought to mimic *B. philenor* (Porter 1989). However, its palatability has never been tested, so it is not known what type of mimicry is operating between this subspecies and *B. philenor*. Finally, the black, white, and orange dorsal wing surface of Lorquin's admiral (*L. lorquini*) resembles the dorsal wing surface of the California sister (*Adelpha bredowii*) and appears to be a Batesian mimic of that species (Porter 1989; Prudic et al. 2002). The presence of five closely related butterfly subspecies which are all mimics, but which resemble vastly different models or co-mimics is remarkable, and much of the interest in this genus has focused on this phenomenon (Mullen 2006; Platt 1983).

Limenitis archippus and *L. arthemis astyanax* butterflies are broadly sympatric throughout most of the eastern United States (Platt 1983). Even though they share the same habitats, they rarely interbreed in the wild (Covell 1994). *L. archippus* shows this same pattern with each of the other species in the genus, while *L. arthemis astyanax* hybridizes freely with all other forms of *Limenitis* (Marcus et al. ms). There are four species of *Limenitis* recognized in North America: *L. arthemis, L. archippus, L. weidemeyerii*, and *L. lorquini* (Platt 1983). All species of *Limenitis* except for *L. archippus* are mostly allopatric and hybridize extensively in areas where their ranges

overlap (Remington 1968; Platt 1983; Porter 1989; Porter 1990). As infrequent as it may be, *L. archippus* and *L. arthemis astyanax* do interbreed in the wild giving rise to a hybrid butterfly called *Limenitis archippus* X *L. arthemis astyanax* form *rubidus*, (subsequently referred to as "rubidus"; Figure 3) (Strecker 1878; Ritland 1990).

Rubidus hybrids resemble both *L. archippus* and *L. arthemis astyanax* (Figure 4). Like *L. archippus*, the ground color of *rubidus* is orange while the dorsal forewings are darkly pigmented like *L. arthemis astyanax* (Platt & Greenfield 1971). The hind wings are distinct with marginal red-orange spots and the ventral side of *rubidus* contains the proximal and marginal red-orange spotting characteristic of *L. arthemis astyanax* (Platt & Greenfield 1971). Platt (1975) conducted experimental hybridizations of *L. archippus* with *L. arthemis astyanax* in the laboratory. All of the crosses and backcrosses that were performed made use of the hand-pairing technique (Platt 1969). Hand-pairings between *L. archippus* and *L. arthemis astyanax* resulted in F_1 male hybrids that were phenotypically intermediate and referable to hybrid form *rubidus* Strecker (Platt 1975). These hybrids consisted of light forms that more closely resembled *L. archippus* and dark forms that more closely resembled *L. arthemis astyanax* (Platt 1975). These interspecific crosses generally do not generate any female butterflies (Platt and Harrison 1994). The F_1 males were then backcrossed to females of the parental species (Platt 1975). The progeny from these backcrosses consisted of phenotypes that were similar to the parental species and phenotypes that were similar to the hybrid form, and very few individuals displayed an intermediate phenotype (Platt 1975; Platt and Harrison 1994).

Natural hybridization between *L. archippus* and *L. arthemis astyanax* is generally rare, but has been observed with some frequency in Hickman, Kentucky (Covell 1994).

Hickman is a city located in Fulton County, the southwestern-most county in the state of Kentucky and is adjacent to the Mississippi River. The viceroy, *L. archippus*, is very abundant at this location, occurring at a frequency that is 10-30 times more abundant than its congener, the red-spotted purple, *L. arthemis astyanax* (Covell 1994). Only 7 heterospecific pairings between the parental species and 72 *rubidus* individuals have been documented in the wild in all of North America (Marcus et al. ms). Of these, 2 heterospecific mating pairs and 2 *rubidus* individuals have been collected along a single 100 meter stretch of the Mississippi River (adjacent to the Bunge Corporation grain elevator in Hickman, Kentucky, GPS coordinates N 36.5661, W -89.2177) over the last several years, suggesting that Hickman, Kentucky may be a "hybridization hotspot" (Covell 1994). A hybridization hotspot is an area where hybridization occurs more frequently than in other areas.

A second, much larger, hybridization hotspot has been described from Northern Florida and Southern Georgia (Ritland 1990). Seven *rubidus* hybrids and two interspecific matings between *L. archippus* and *L. arthemis astyanax* were documented in this area within a two-year period (Ritland 1990). Why are these populations of *L. archippus* and *L. arthemis astyanax* in Florida, Georgia, and Kentucky hybridizing so frequently? Ritland proposed two explanations for the frequent hybridization of populations in Florida and Georgia (Ritland 1990). The first explanation is that matings between *L. archippus* and *L. arthemis astyanax* occur more frequently in this area of Florida/Georgia than anywhere else and pre-mating reproductive isolation is drastically reduced (Ritland 1990). The second explanation is that matings between *L.* archippus and *L. arthemis astyanax* are not more frequent in this area of Florida/Georgia but the

hybrids that are produced are more viable than those produced elsewhere (Ritland 1990). According to this explanation, post-mating reproductive isolation is also drastically reduced (Ritland 1990). Ritland then proposed three reasons why pre-mating isolating mechanisms would break down in this area and allow more frequent matings between *L. archippus* and *L. arthemis astyanax* (Ritland 1990).

(1) Habitat overlap

L. archippus and *L. arthemis astyanax* eat different larval foodplants (Remington 1968). *L. archippus* feeds on willows and poplars (*Salix* and *Populus*, Salicaceae), while *L. arthemis astyanax* feeds on black cherry (*Prunus*, Rosaceae) (Remington 1968). These plants do not usually grow in the same habitats so the larvae that feed on them are usually segregated (Shapiro & Biggs 1968). This habitat segregation is one of the mechanisms of pre-mating isolation that has been suggested (Platt et al. 1978). However, in this area of Florida/Georgia *L. arthemis astyanax* occasionally feed on willow instead of black cherry (Ritland 1990). This switch from willow to black cherry allows *L. archippus* and *L. arthemis astyanax* to encounter each other more frequently in this habitat than elsewhere (Ritland 1990). Habitat overlap has been known to increase hybridization between species that are normally segregated (Chapin 1948; Anderson 1949; Mayr 1963; Williams 1983).

(2) Economics of *Limenitis arthemis astyanax* mate choice

In the northern Florida/southern Georgia habitat *L. archippus* outnumbers *L. arthemis astyanax* by a ratio of 9:1 (Ritland 1990). As a result, *L. arthemis astyanax* may have difficulty finding a conspecific mate and end up mating with *L. archippus* because they are the only available option (Ritland 1990). Hybridization has been known to increase

when one species is rare in comparison to the other species (Hubbs 1955; Mayr 1963; Wittenberger 1983).

(3) Biogeography and genetics

In northern Florida *L. arthemis astyanax* encounters *L. archippus* that differ in mate choice behavior from the *L. archippus* that they encounter in other places (Ritland 1990). The areas in Georgia and Florida where *rubidus* individuals were collected correspond geographically with an intraspecific hybrid zone between two subspecies of *L. archippus* (Ritland 1990). This hybrid zone allows *L. archippus archippus* (Cramer) to join with *L. archippus floridensis* (Strecker) and is characterized by a gradual change in the latitudinal wing color (Ritland 1990). *Limenitis archippus archippus* is broadly sympatric with *L. arthemis astyanax*, while *L. archippus floridensis* inhabits an area of Florida that is largely isolated from other species of *Limenitis* (Ritland 1990). This may allow *L. archippus floridensis* to be less discriminating when choosing a mate (Ritland 1990).

Two of the three factors that might contribute to the break down of pre-mating isolating mechanisms identified by Ritland (1990) could be playing a role at the Hickman, Kentucky site. First, there are abundant willow trees observed at the site, while black cherry is not present, so larvae of both species may be feeding on the same host plant (Covell 1994). Second, there were more *L. archippus* observed at the site than *L. arthemis astyanax* outnumbering the second species by a ratio of 10:1 (Covell 1994) to 30:1 (Jeffrey Marcus and William Black, Jr., unpublished data, 2005). However, Hickman, Kentucky is outside of the *L. archippus* hybrid zone (Platt 1983), so the possible lack of mate discrimination by *L. archippus floridensis* is unlikely to play a role at this location.

Increased hybridization between *L. archippus* and *L. arthemis astyanax* in Hickman, Kentucky may allow for a limited amount of genetic introgression between the two species (Anderson 1949). Introgression is the movement of a gene from one species into the gene pool of another by backcrossing an interspecific hybrid with one of its parents. Introgression between *L. archippus* and *L. arthemis astyanax* is possible because Platt's laboratory experiments showed that *rubidus* males are fertile in backcrosses to both parental species (Platt 1983). If this is the case, then introgression could affect a number of traits in both species of *Limenitis*.

I am interested in the extent to which hybridization has allowed gene flow between *L. archippus* and *L. arthemis astyanax* at this location. Twelve individuals of each species were collected at Hickman, Kentucky. Two different techniques were used to study these samples. Initially, specimens were analyzed for mitochondrial DNA haplotype using DNA sequencing techniques. Mitochondrial sequences in Lepidoptera are useful when looking at the genetic divergence between species for two reasons (Mallet et al. 2007). First, due to unisexual inheritance, there is thought to be no recombination between mitochondria (Mallet et al. 2007). As a result, genetic divergence is not likely to be affected by occasional introgression (Mallet et al. 2007). Second, in many species of Lepidoptera, hybrid females are usually sterile, in accordance with Haldane's rule (Presgraves 2002). Haldane's rule ensures that the introgression of mitochondria is prevented at an earlier stage of speciation, and that nuclear loci can be transferred between species by backcrossing male hybrids (Jiggins et al. 2001; Sperling 1990; Naisbit et al. 2002).

Specimens were also analyzed using a technique called Randomly Amplified DNA Fingerprints (RAF) (Schlipalius et al. 2001; Waldron et al. 2002). RAF is a procedure that can be readily used for population studies and Mendelian genetic studies (Schlipalius et al. 2001). One of the benefits of applying RAF is that it works with insects of any size (Schlipalius et al. 2001). RAF has several advantages over previously established procedures for the generation of polymorphic DNA markers including: robustness, reliability, no requirement for a highly-purified DNA template, few steps, sensitive detection with radio-labeling or fluorescent tagging, simultaneous detection of several markers, and the identification of codominant loci (Waldron et al. 2002). In this study, RAF was employed to assess the nuclear genetic diversity between *L. archippus* and *L. arthemis astyanax* from Hickman, Kentucky.

MATERIALS AND METHODS

(a) Molecular characterization and Sequencing of *Limenitis archippus* and *Limenitis arthemis astyanax*

Limenitis archippus (viceroys) and *L. arthemis astyanax* (red-spotted purples) individuals were collected from the bait traps at the Bunge Site, Hickman, KY on September 11, 2005. Twelve individuals of each species were collected in total. These specimens were transported alive at 4°C to the lab at Western Kentucky University where they were stored at -20°C. Tissue from the abdomen of each wild-caught specimen was harvested and DNA was extracted using the QIAGEN DNEasy kit. PCR was used to amplify a portion of the mitochondrial COI gene using the universal primers LCO 1490 and HCO 2198 (Table 1) (Folmer et al. 1994). The reaction mixture for each sample contained: 1μL of DNA (Table 2), 1μL of LCO 1490 primer (Table 3), 1μL of HCO 2198 primer (Table 3), 9.5μL of Nanopure water, and 12.5μL of Quick-Load Master Mix (New England Biolabs). The PCR conditions were as follows: one cycle of 95°C for 5 minutes, thirty-five cycles of 94°C for 1 minute, 46°C for 1 minute, 72°C for 1.5 minutes, and one cycle of 72°C for 5 minutes, and 4°C indefinitely in a BioRad MyCycler Thermocycler. This primer set is expected to produce a PCR product of 650 base pairs after the PCR reaction. The size of the amplification products was checked using agarose gel electrophoresis. Successful amplifications were used for sequencing reactions in both directions using the LCO 1490 and HCO 2198 primers, Big Dye Fluorescent sequencing reagents, and a BioRad MyCycler Thermocycler. The reaction mixture

for each sample contained: 4.5μL of Nanopure water, 2.5μL of Sequencing Buffer, 0.5μL of primer (2 reactions for each PCR product, one with forward primer and one with reverse primer), 2μL of PCR product, and 1.5μL of Big Dye Terminator v3.1 Cycle Sequencing Mix (Applied Biosystems). The PCR conditions were as follows: 25 cycles of 96°C for 30 seconds, 50°C for 15 seconds, and 60°C for 4 minutes, and one cycle of 10°C indefinitely. After cleanup by isopropanol precipitation, sequences were loaded into an ABI 3130 automated sequencer. The resulting sequences were edited in Sequencher 4.5 (Sequencher 2005) and then aligned using Clustal W (Thompson et al. 1994). Finally, phylogenetic trees were produced using PAUP* 4.0610 (Swofford 1998). Both Neighbor-Joining and Parsimony methods were used to reconstruct the phylogeny based on the variation among the mitochondrial haplotypes.

(b) Randomly Amplified Fingerprints (RAF) of *Limenitis archippus* and *Limenitis arthemis astyanax*

Of the initial 12 specimens of *L. archippus* and *L. arthemis astyanax* collected, usable *cytochrome oxidase I* sequences were obtained from 10 specimens from each species. These 20 individuals were analyzed by Randomly Amplified Fingerprints (RAF) (Schlipalius et al. 2001; Waldron et al. 2002). Two replicates of each *Limenitis* sample were amplified by RAF. This technique was employed to examine the nuclear DNA diversity between *L. archippus* and *L. arthemis astyanax*. Multiple blanks containing no template DNA were also amplified to serve as a negative control. Six different oligonucleotide primers were used, including: RP2, RP4, RP6 (designed by Schlipalius et al. 2001), MRJ-1, MRJ-2, and MRJ-3 (designed for this

study, Table 1). The reaction mixture for each sample contained: 1μL of DNA (Table 2), 4μL of Quick-Load Master Mix (New England Biolabs), and 5μL of primer (Table 3). PCR conditions were as follows: one cycle of 94°C for 5 minutes, thirty cycles of 94°C for 30 seconds, 57°C for 1 minute, 56°C for 1 minute, 55°C for 1 minute, 54°C for 1 minute, 53°C for 1 minute, and one cycle of 72°C for 5 minutes, and 4°C indefinitely in a BioRad MyCycler Thermocycler. The samples were analyzed on an ABI 3130 sequencer with 1uL of Rox-500 Gene Scan Size Standard and 10uL of HiDye Formamide (Applied Biosystems). Using Genemapper, bands that appear in both the Nanopure water negative controls and in the experimental samples were identified and eliminated from the samples because they represent experimental artifacts, which are not useful during analysis. We scored each individual for the presence or absence of RAF bands using binary code. If two replicates of the same sample showed the same band they received a 1. To be conservative, if two replicates of the same sample did not show the same band they received a 0. The resulting data were used to create a phylogenetic data matrix. All of the phylogenetic analyses were performed with the computer program PAUP* 4.0610 (Swofford 1998). The first analysis performed was a parsimony-based heuristic search for the best trees. A heuristic search was used with 1,000 random taxon addition replicates. Next, the data were analyzed by a parsimony analysis with 1,000,000 bootstrap replicates with a simple taxon addition sequence for each. This analysis was performed to assess the statistical confidence of each node using data subsampling. Groups that were compatible with 50% majority-rule consensus were

retained in the final consensus tree. The final test performed was an Incongruence Length Difference (ILD) test. The ILD is the difference between the number of steps required by the individual partitions to generate a tree topology and the number of steps it takes for the combined partitions to generate the same topology. The ILD test compares the ILD statistic of the specified partitions of informative characters with the ILD for a series of randomized partitions of the same sizes as the original partitions, but represents a mixture of characters from each partition (Cunningham 1997). This test was performed in PAUP* (Swofford 1998) as the partition-homogeneity test with 1,000 replicates and a "maxtrees" setting of 200 trees per replicate in order to reduce the analysis time. We used a pairwise comparison approach, examining all of the datasets in pairs to see which ones were heterogeneous with each other.

RESULTS

(a) Molecular characterization and Sequencing of *Limenitis archippus* and *Limenitis arthemis astyanax*

Limenitis archippus (viceroy butterfly) and *L. arthemis astyanax* (red-spotted purple butterfly) individuals were collected from the bait traps at the Bunge Site, Hickman, KY on September 11, 2005. Twelve individuals of each species were collected in total. PCR was used to amplify a portion of the mitochondrial COI gene using the universal primers LCO 1490 and HCO 2198. For each species, only 10 out of 12 PCR amplifications were successful, for a total of 20 amplifications. I sequenced the mitochondrial gene COI from the twenty successful amplifications in both directions, using the same primers used for PCR amplification. The sequences were edited by consulting chromatograms in Sequencher (Sequencher 2005), and then aligned in Clustal W (Thompson et al. 1994). Alignments were converted into NEXUS format for further analysis in PAUP* (Swofford 1998). The alignments were analyzed using the Neighbor-joining (Figure 5) and parsimony (Figure 6) settings of PAUP* (Swofford 1998), both of which grouped the members of each species into a separate monophyletic group. In other words, mitochondrial DNA sequences could be used to distinguish between the two species of butterflies.

(b) Randomly Amplified Fingerprints (RAF) of *Limenitis archippus* and *Limenitis arthemis astyanax*

Each individual *Limenitis* was scored for the presence or absence of RAF bands using ABI Genemapper software (Figure 7). The number of bands recovered for each of the six primers was variable, with some primers producing a large amount of bands and other

primers producing very few bands (Table 4). The resulting data were used to create a phylogenetic data matrix. The first analysis performed was a parsimony-based heuristic search for the best trees. This heuristic search resulted in one most parsimonious tree (Figure 8). The tree groups several *Limenitis arthemis astyanax* individuals, RSP6, RSP1, RSP4, RSP7, RSP3, and RSP11, with *L. archippus*, suggesting that some gene flow between these species may be occurring.

To assess the statistical confidence of this pattern a bootstrap analysis with 1,000,000 bootstrap replicates was performed with a simple taxon addition sequence for each. Groups that were compatible with 50% majority-rule consensus were retained in the final consensus tree. A Bootstrap analysis assesses the statistical confidence of each node using repeated data subsampling. The resulting tree (Figure 9) does not show strong evidence for or against hybridization, because the statistical confidence at most nodes is less than 50%.

The final test performed was an Incongruence Length Difference (ILD) test. The ILD is the difference between the number of steps required by the individual partitions to generate a tree topology and the number of steps it takes for the combined partitions to generate the same topology. The ILD test compares the ILD statistic of the specified partitions of informative characters with the ILD for a series of randomized partitions of the same sizes as the original partitions, but represents a mixture of characters from each partition (Cunningham 1997). The results of the ILD test show that many of the partitions are incongruous with one another (Table 5), suggesting that there is no one partition that is responsible for the production of conflicting data in the bootstrap analysis described above.

DISCUSSION

Little is known about the evolutionary significance of hybridization and introgression in animals, even though evidence has shown that they both occur with some frequency (Bernatchez et al. 1995). In contrast, plant hybrids may play an important role in the formation of new species (Rieseberg & Willis 2007). Animal hybrids are considered rare on a per individual basis, but recent studies have shown that many species hybridize (Coyne & Orr 2004; Mallet 2005). Nearly 10% of animal species and 25% of plant species hybridize in the wild, while in some animal groups such as ducks, birds of paradise, and *swallowtail* butterflies, 40-75% of species are known to hybridize (Mallet 2005).

In the past, the transfer of genes across species boundaries was thought to have little or no evolutionary importance (Mayr 1963). Recently, it has been brought to light that introgressive interspecific hybridization can have significant and long-term effects on an organism's genetic composition (Bernatchez et al. 1995). For example, one of the explanations proposed by Ritland (1990) for the occurrence of hybridization between *Limenitis archippus archippus* and *L. arthemis astyanax* in Georgia and Florida is that there has been introgression of genetic factors from *L. archippus floridensis* into *L. archippus archippus*. This introgression may be responsible for making this population of *L. archippus archippus* less discriminating. Despite the many studies and surveys that have been performed to date, assessing the magnitude of the genetic impact of hybridization and introgression still remains an unresolved issue in evolutionary biology.

Hybridization between *L. archippus* and *L. arthemis astyanax* in Hickman, Kentucky may allow for a limited amount of genetic introgression between the two

species (Anderson 1949). If this is the case, then introgression could affect a number of traits in both species of *Limenitis* such as larval host plant preference, mate preference, and color pattern characteristics. All of these traits could influence the ability of these populations to continue to participate in mimicry complexes.

In the present study, I determined the extent to which hybridization has allowed gene flow between *L. archippus* and *L. arthemis astyanax* at this location. Two different techniques were used to study this unique butterfly population. Initially, specimens were analyzed for mitochondrial DNA haplotype using DNA sequencing techniques and then RAF was employed to assess the nuclear genetic diversity between *L. archippus* and *L. arthemis astyanax.*

Analysis of COI sequences sorts the specimens unambiguously into the two species of *Limenitis* butterflies present in Hickman, Kentucky (Figure 5 and 6). This suggests that while hybridization may be occurring at this location (Covell 1994; Marcus et al. ms), it is not resulting in extensive introgression of mitochondrial haplotypes between these two species.

Phylogenetic analysis of the RAF data suggests that it is possible that there is some genetic exchange between these two species. In the parsimony analysis of the entire RAF data set, one *L. arthemis astyanax* specimen, RSP6, clearly shows more affinity to the nuclear DNA fingerprint patterns of *L. archippus* than to members of its own species and an additional group of *L. arthemis astyanax* specimens (RSP1, RSP3, RSP4, RSP7, and RSP11) is within the clade that includes all of the *L. archippus* specimens (Figure 8). However, a bootstrap analysis of the entire RAF data set shows that the statistical support for these relationships is very weak and dependent on just a

small number of characters. In fact, most specimens group according to species in the bootstrap tree (Figure 9). The ILD Test was used to determine if the weak bootstrap support could be attributed to markers from one RAF primer that was somehow conflicting with the data produced by the other primers. However, pair-wise comparisons between each of the RAF primer data sets show that 4 out of the 6 primers (RP2, RP4, MRJ-1, and MRJ-2) are heterogeneous with respect to each other. This suggests that rather than one incongruous data partition reducing tree resolution, the entire data set is incongruous and the lack of resolution cannot be attributed to the data from a single primer. Since RAF primers generate markers from all of the chromosomes simultaneously, when introgression is occurring, one might expect that even data produced by a single RAF primer might show incongruity because some markers originate from one parental species, while some markers originate from the other parental species. On the whole, the RAF data suggest that while there may be some movement of nuclear markers between these two species, it is not yet possible to say with statistical confidence that this is the case. Interestingly, only some individuals of the rarer of the two species at Hickman, *L. arthemis astyanax*, show any evidence of genetic introgression, suggesting that if introgression is occurring, there may be an asymmetrical movement of these nuclear markers at the Hickman, Kentucky site.

A study with some similarities to this one was performed by Bernatchez et al. (1995) on a population of brook trout, *Salvelinus fontinalis*, from Lake Alain in Québec. The mitochondrial genotype of 48 *S. fontinalis* was characterized by RFLP analysis performed on the entire mitochondrial DNA molecule (Bernatchez et al. 1995). The 48 fish examined were morphologically indistinguishable from typical brook trout and were

homozygous for the alleles characteristic of brook trout (Bernatchez et al. 1995). The results of this study showed that the mitochondrial DNA of all 48 *S. fontinalis* individuals was identical to the haplotype of Québec Arctic char, *Salvelinus alpinus* (Bernatchez et al. 1995). The permanent replacement of *S. fontinalis* mitochondrial DNA with that of *S. alpinus* could be selectively significant. Several mitochondrial enzymes that are central to intermediary metabolism are encoded by mitochondrial genes, allowing differences in the mitochondrial genome of these fish to be manifested physiologically (Bernatchez et al. 1995). Genetic differences between the mitochondrial genomes of introgressed populations, like that previously mentioned, can translate to differences in the metabolic capacity of the mitochondria. Varying metabolic capacities can lead to differences in adaptivity to environmental temperature (Bernatchez et al. 1995).

Similarly, Good et al. (2008) describe hybridization between two species of chipmunk from western North America. The yellow-pine chipmunk, *Tamias amoenus*, inhabits xeric forests across western North America (Sutton 1992), while the red-tailed chipmunk, *T. ruficaudus*, primarily inhabits mesic forests of the northern Rocky Mountains (Best 1993). *Tamias amoenus* and *T. ruficaudus* occupy different ecological niches and have distinct genital bone morphologies, and yet are not completely reproductively isolated in areas of sympatry (Good et al. 2008). The researchers were looking to find the extent and pattern of introgression across both mitochondrial and nuclear loci in both taxa (Good et al. 2008). They examined the genetic variation at one mitochondrial locus and 11 nuclear loci. Their findings suggest that there is extensive asymmetric introgression of mitochondrial DNA from *T. ruficaudus* into *T. amoenus* with comparatively little introgression at the nuclear loci (Good et al. 2008). Overall,

introgression has had a minimal impact on the nuclear genomes of these two species of chipmunk, despite multiple independent hybridization events (Good et al. 2008).

In contrast, studies conducted by Halbert and Derr (2007) show that introgression of cattle genes has a very different pattern in populations of United States bison. In the late 1800's the North American bison, *Bison bison* was near extinction (Cunningham & Berger 1994). Thanks to the efforts of a few individuals, who captured and raised them in zoos and on private ranches, the population of North American bison has been restored (Cunningham & Berger 1994). These efforts are now threatened by introgression with domestic cattle, because hybrid species are not protected by the Endangered Species Act (O'Brien & Mayr 1991). Eleven populations of bison from the United States were examined for evidence of mitochondrial and nuclear introgression with domestic cattle, *Bos taurus* (Halbert & Derr 2007). Mitochondrial introgression was assessed using PCR and analysis of D-loop sequences, while nuclear introgression was assessed in 14 chromosomal regions by examining microsatellite electromorph and sequence differences between the two species (Halbert & Derr 2007). Their findings show one population of bison with domestic cattle mitochondrial DNA introgression, while nuclear introgression was found in seven of the bison populations (Halbert & Derr 2007).

Studies were conducted by Dasmahapatra et al. (2007) on hybridizing non-sister species of *Heliconius* butterflies. Although interspecific hybridization occurs regularly in wild *Heliconius* butterflies, hybrid individuals are rare (Dasmahapatra et al. 2007). This is the first study where two distantly related *Heliconius* species have been genetically examined. Dasmahapatra et al. (2007) used molecular markers to determine the parents of a hybrid butterfly that was captured in Peru. Mitochondrial and nuclear genes

indicated that the specimen was an F_1 hybrid between a female *Heliconius ethilla* and a male *Heliconius melpomene* (Dasmahapatra et al. 2007). The presence of such distant natural hybrids, along with evidence of backcrossing suggests that gene flow across the species boundary can take place long after speciation has occurred (Dasmahapatra et al. 2007). Within the *melpomene-cydno* group, hybridization and backcrossing has led to interspecific introgression at several genomic regions (Bull et al. 2006; Kronforst et al. 2006). If extensive hybridization among two closely related species can cause adaptive genes to introgress, then rarer hybridization between two distantly related species may also play a role (Dasmahapatra et al. 2007). As a result, adaptive genes, like those involved in wing coloration, could be widely shared among members of this highly mimetic genus (Dasmahapatra et al. 2007).

Mallet et al. (2007) took *Heliconius* research one step further by analyzing all known cases of interspecific hybridization in Heliconiina. It has been determined that hybridization is a naturally occurring phenomenon between species of *Heliconius* (Jiggins et al. 1997). Previous work has shown that backcrossing occurs in the wild and that backcrosses performed in the lab are fertile (Jiggins et al. 2005; Kapan et al. 2006), which would allow for genetic introgression. Genetic introgression may contribute to both adaptive evolution and speciation. Molecular work performed on hybridizing sympatric species of *Heliconius* shows that alleles at several loci have been exchanged between species (Mallet et al. 2007). Based on a molecular clock, gene exchange can continue for more than 3 million years after speciation. This study's findings agree with the idea that processes leading to speciation are continuous, and that they are the same as those processes operating within species (Mallet et al. 2007). Furthermore, adaptive

genes can be transferred, playing an important role in both adaptation and speciation (Mallet et al. 2007).

In the studies conducted by Bernatchez et al. (1995) and Good et al. (2008), mitochondrial introgression was occurring, while the nuclear genome was minimally affected. Conversely, the studies conducted by Halbert and Derr (2007), Dasmahapatra et al. (2007), and Mallet et al. (2007) showed that nuclear introgression was occurring at a high frequency, while mitochondrial introgression was occurring at a much lower frequency. My study showed that hybridization between two sympatric species of *Limenitis* butterflies in Hickman, Kentucky led to no introgression of the mitochondrial DNA and suggested that a limited amount of nuclear introgression may be occurring due to hybridization, making it similar to the second group of studies. The *L. archippus* lineage diverged from the *L. arthemis astyanax* lineage about 1.4 million years ago (Marcus et al. ms), placing *Limenitis* within the 3 million year post-speciation range suggested by Mallet et al (2007) as the period when genetic exchange can still occur.

Studies like those previously mentioned show that introgressive interspecific hybridization can have significant and long-term effects on an organism's genetic composition, and in order to fully understand the significance of hybridization and introgression among animal species, we must continue researching and collecting information on these subjects. In the case of *Limenitis*, it would be particularly advantageous to use additional techniques for the study of nuclear genetic markers to try and better-document the possible introgression that is occurring. The anonymous markers generated by RAF were not as informative as I had hoped. It might be advisable for future studies to focus on single nucleotide polymorphisms (SNPs), microsatellites,

and other markers that are attributable to specific genetic loci in order to not only document introgression, but also to specify exactly which components of the nuclear genome are introgressing.

Figure 1 a. Viceroy, *Limenitis archippus*, Hickman, Kentucky b. Red-spotted Purple, *Limenitis arthemis astyanax*, Hickman, Kentucky c. Monarch, *Danaus plexippus*, Paducah, Kentucky d. Pipevine swallowtail, *Battus philenor*, Upper Green River Biological Preserve, Hart County, Kentucky (images a-c photographed by Dr. Jeffrey Marcus, 2003; image d photographed by Dr. John Andersland, 2003)

Figure 2 Female *Limenitis archippus* (left) and male *Limenitis arthemis astyanax* (right) mating in Hickman, Kentucky, September 6, 1997. (Photographed by Dr. Charles V. Covell Jr.)

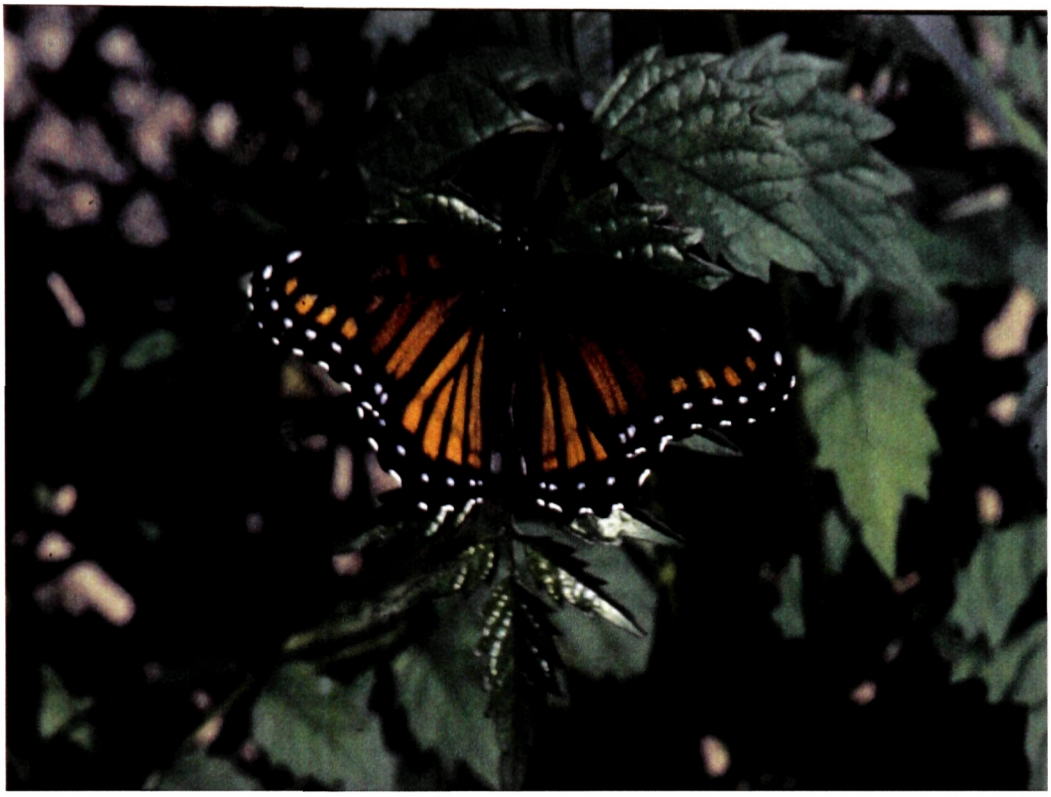

Figure 3 *Limenitis archippus* X *Limenitis arthemis astyanax* f. *rubidus* hybrid from Hickman, Kentucky, September 7, 2002. (Photographed by Dr. Charles V. Covell Jr.)

Figure 4 a. Dorsal and b. ventral views of a *rubidus* hybrid collected in Hickman, Kentucky, September 14, 1980. Specimen was collected by Dr. Charles V. Covell Jr. and is now in the collections of the McGuire Center for Lepidoptera and Biodiversity in Gainesville, FL.

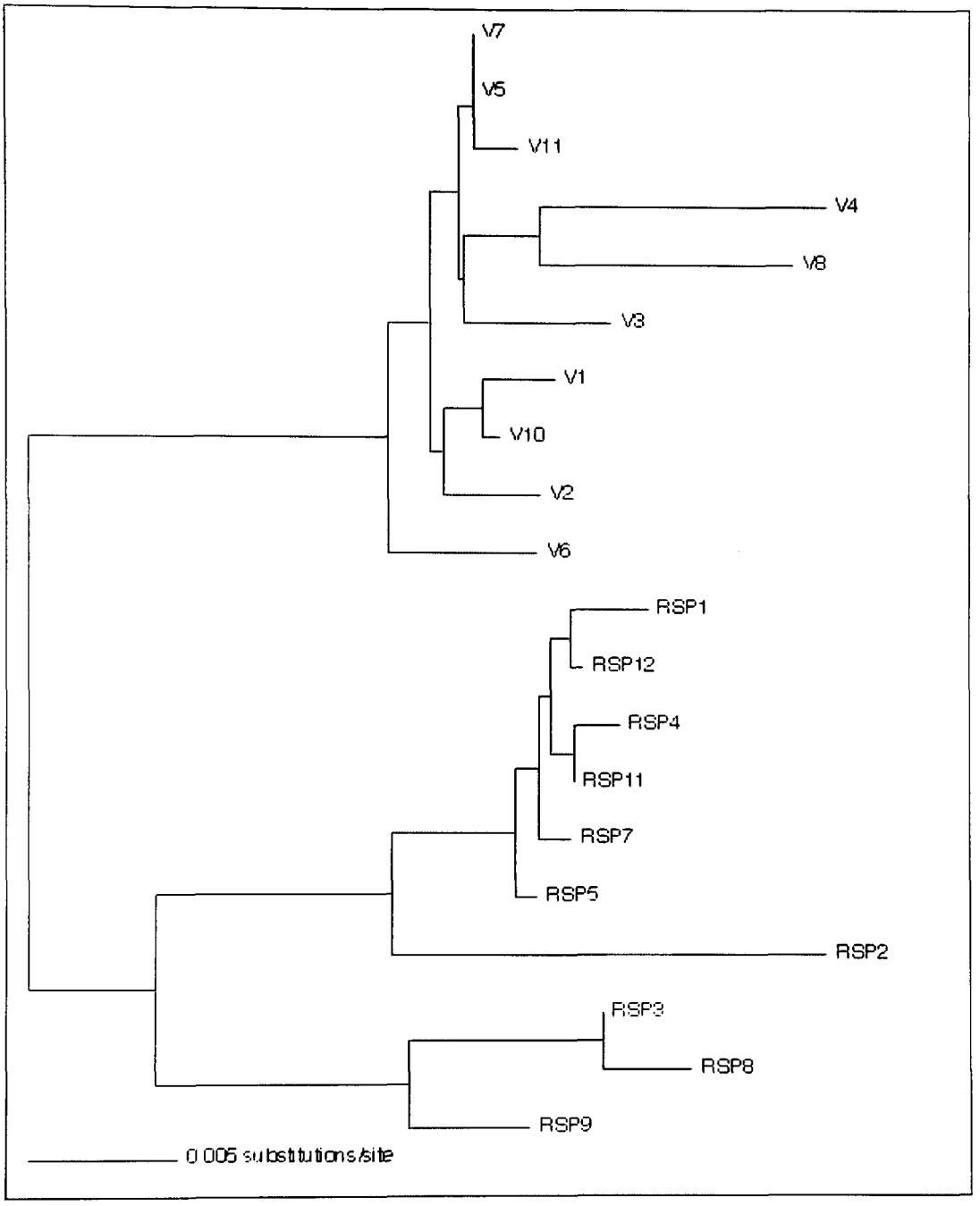

Figure 5 Neighbor-joining tree of Cytochrome Oxidase I sequences. Data aligned by Clustal W and analyzed by PAUP* (Swofford, 1998). Codes V1-V12 refer to viceroys, *L. archippus* while codes RSP1-RSP12 refer to red-spotted purples, *L. arthemis astyanax*.

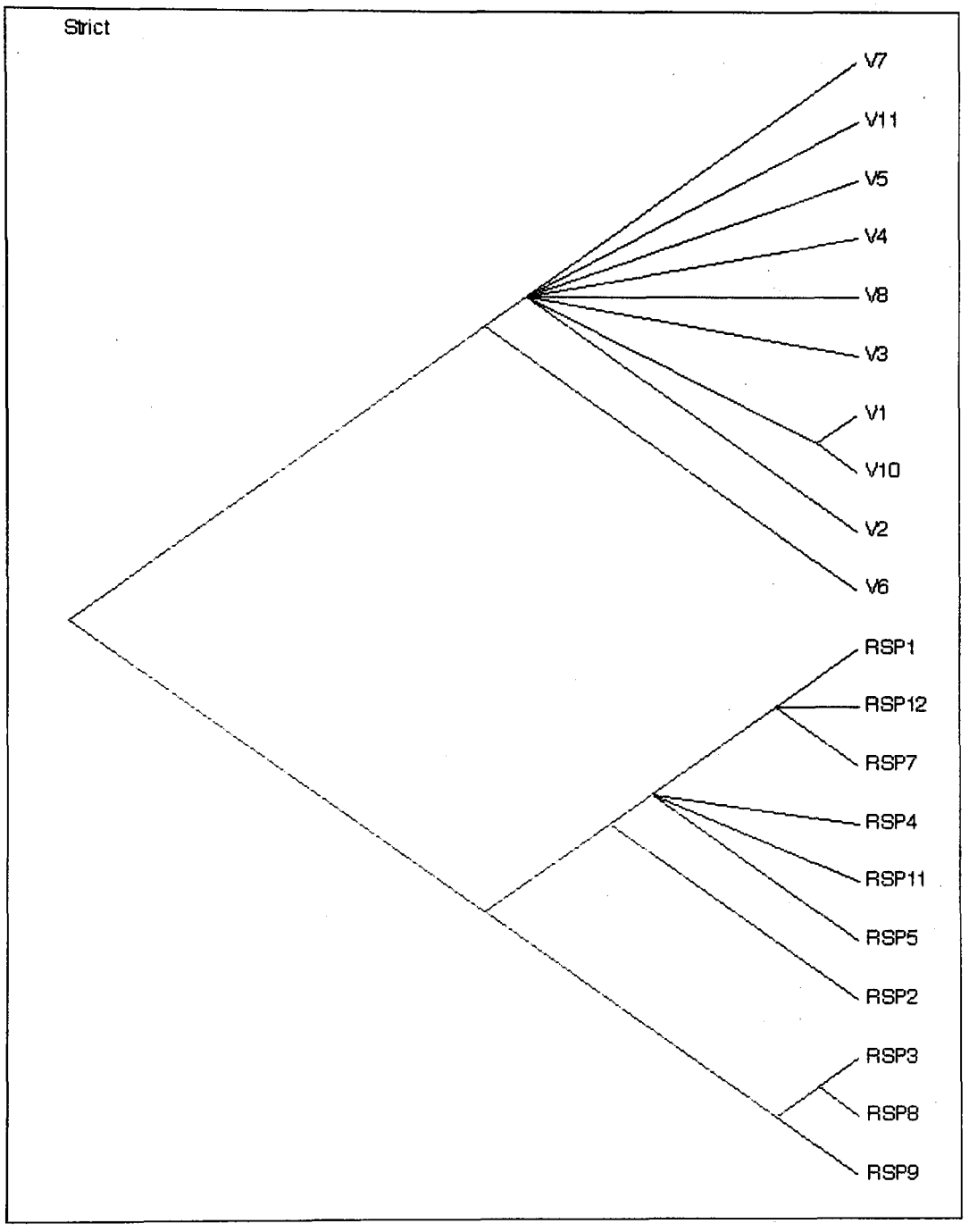

Figure 6 Strict consensus of 10,000 equally most parsimonious trees based on analysis of Cytochrome Oxidase I DNA sequences. Data aligned by Clustal W and analyzed by PAUP* (Swofford, 1998). Codes are the same as described for Figure 5.

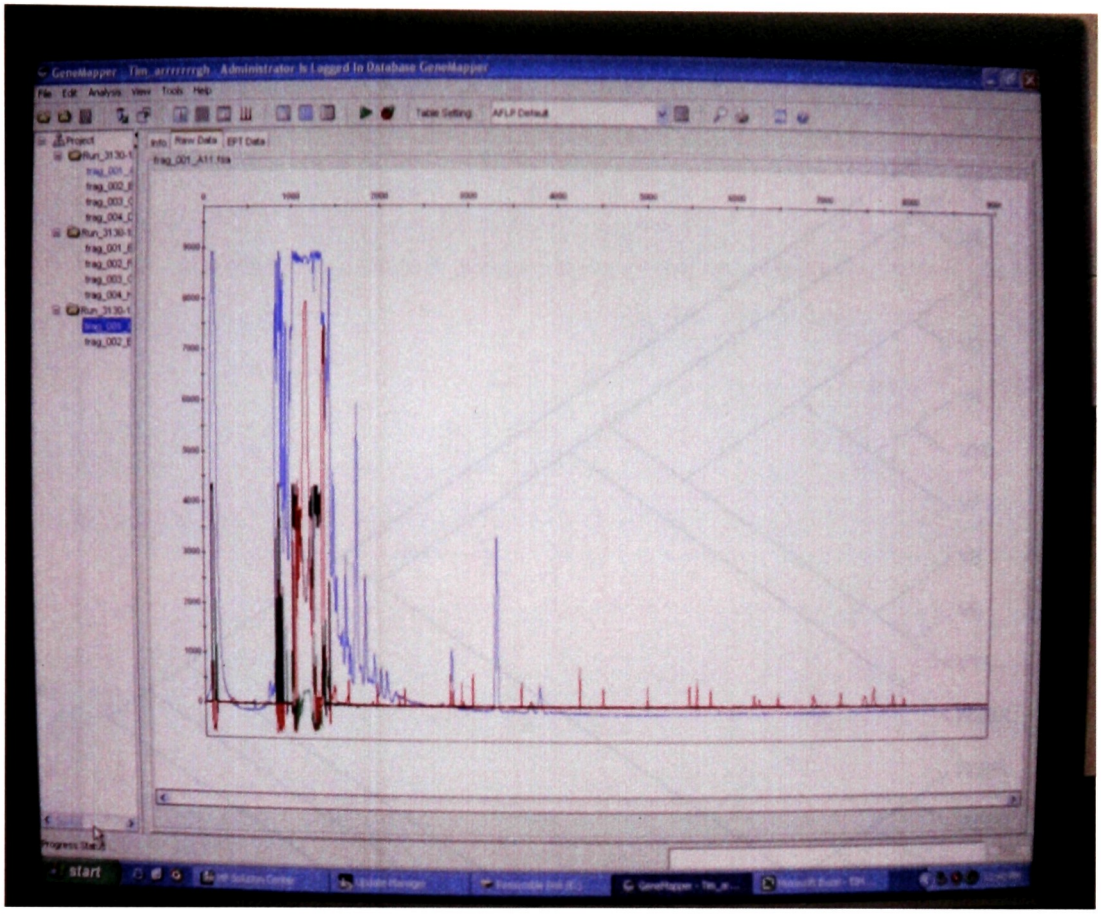

Figure 7 RAF electropherogram

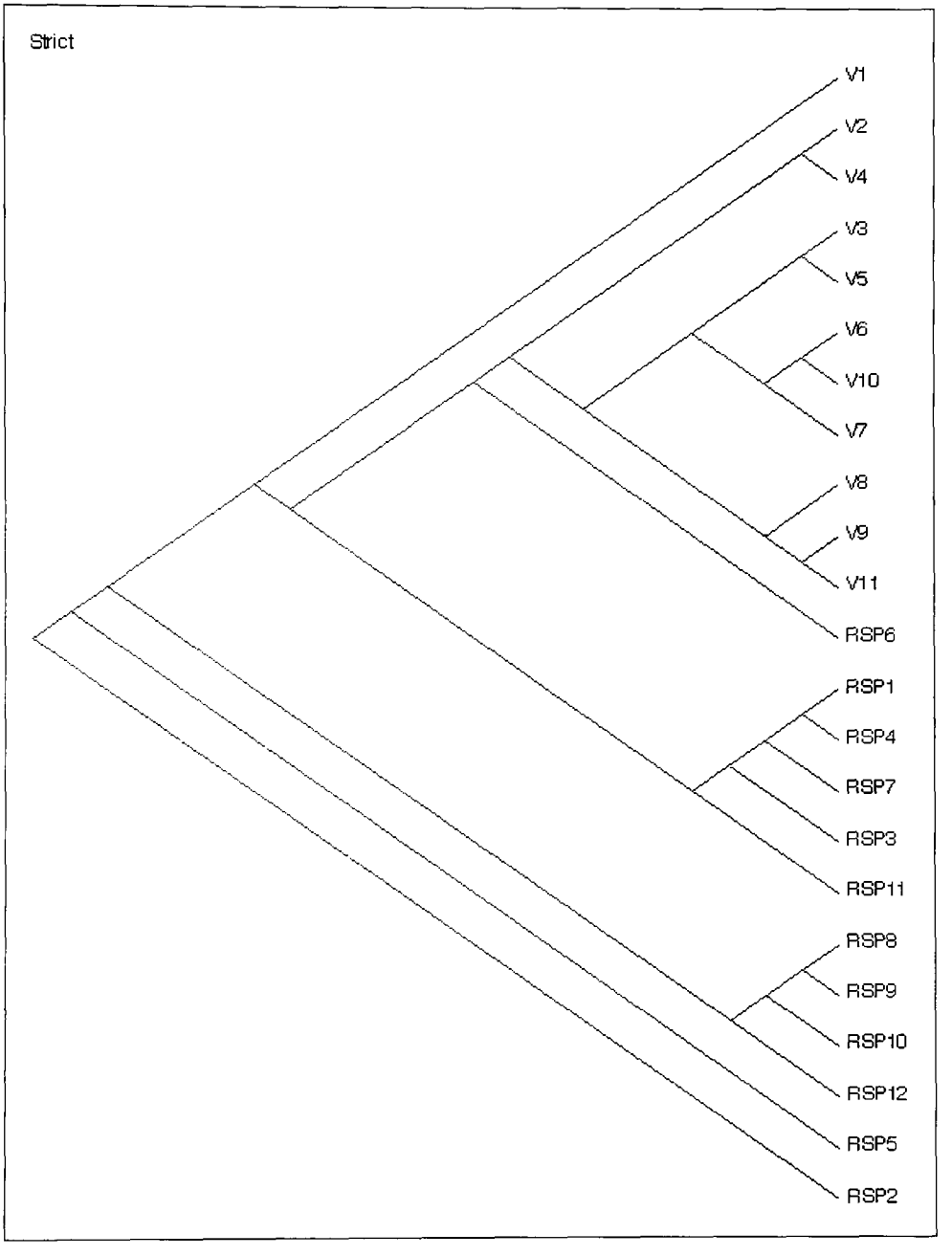

Figure 8 Most parsimonious tree based on analysis of Randomly Amplified DNA Fingerprints (RAF) using RP2, RP4, RP6, MRJ-1, MRJ-2, AND MRJ-3 primers. Data analyzed by PAUP* (Swofford, 1998). Codes are the same as described for Figure 5.

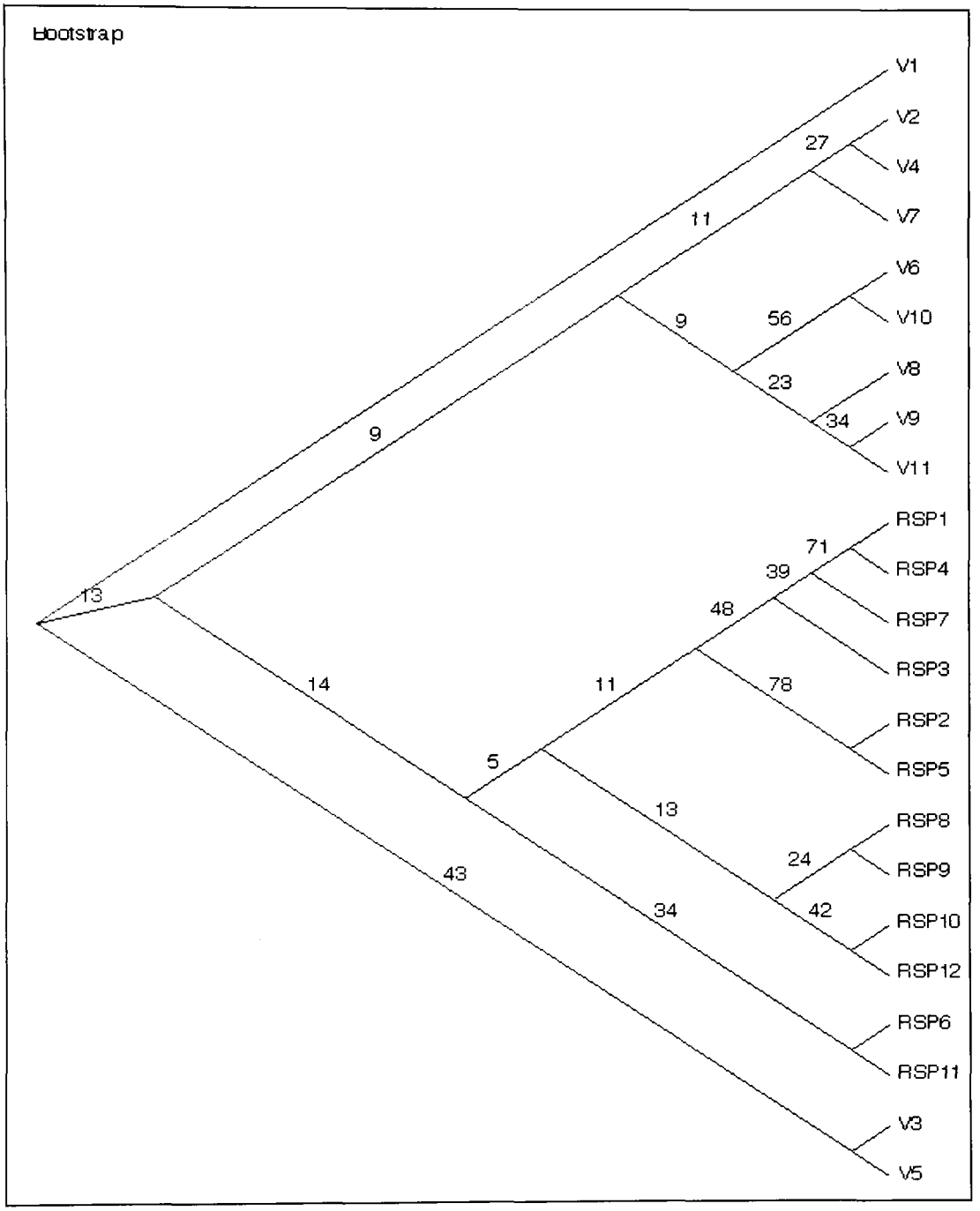

Figure 9 Bootstrap consensus tree, 1,000,000 simple sequence addition repeats of RAF data. Numbers associated with nodes are measures of the statistical confidence supporting that node. Data analyzed by PAUP* (Swofford, 1998). Codes are the same as described for Figure 5.

Table 1: PCR and RAF Primer Sequences

Primer	Sequence
LCO 1490 (f)	GGTCAACAAATCATAAAGATATTGG
HCO 2198 (r)	TAAACTTCAGGGTGACCAAAAAATCA
RP2	ATGAAGGGGTT
RP4	TGCTGGTTCCC
RP6	TGCTGGTTTCC
MRJ-1	CTATTCGAGCC
MRJ-2	ATTCTCCGCAA
MRJ-3	CTTCTCCGCCT

Table 2: Concentration of DNA

DNA Sample	Concentration (ng/μL)
RSP1	291.4
RSP2	202.8
RSP3	257.6
RSP4	267.1
RSP5	84.1
RSP6	334.7
RSP7	113.6
RSP8	224.2
RSP9	104.9
RSP10	323.8
RSP11	113.0
RSP12	351.3
V1	182.4
V2	394.1
V3	1026.0
V4	1334.6
V5	1189.8
V6	822.4
V7	1192.2
V8	755.2
V9	1922.5
V10	787.7
V11	1543.6
V12	122.9

Table 3: Concentration of Primer Stocks

Primer	Concentration
LCO 1490 (f)	23.57 nmol
HCO 2198 (r)	34.30 nmol
RP2	250 nmol
RP4	100 nmol
RP6	100 nmol
MRJ-1	100 μmol
MRJ-2	100 μmol
MRJ-3	100 μmol

Table 4: RAF Bands Recovered for Each Primer

Primer	Number of Bands Recovered
RP2	65
RP4	62
RP6	2
MRJ-1	120
MRJ-2	143
MRJ-3	15

Table 5: Results of Partition Homogeneity Test. Numbers listed are the probabilities (p) that two data partitions are homogeneous with respect to each other. Probabilities less than 0.05 can be considered significant and indicate partitions that are heterogeneous.

	RP2	RP4	RP6	MRJ-1	MRJ-2	MRJ-3
RP2	-	0.001	0.799	0.001	0.001	0.295
RP4	0.001	-	0.708	0.001	0.001	0.138
RP6	0.799	0.708	-	0.832	0.985	1
MR-1	0.001	0.001	0.832	-	0.001	0.147
MRJ-2	0.001	0.001	0.985	0.001	-	0.658
MRJ-3	0.295	0.138	1	0.147	0.658	-

APPENDIX

Appendix Table 1: Binary Code with RP2 Primer

Sample	Binary Code
V1	01100000010101000000010000100010000000011000001100011000000000000
V2	00000000010000000000100000000000000000000000010000100000001100010
V3	01100000000100000000000000110001000000011000000100010001000000010
V4	00000010010000000000000000000000000000000000010101001000000010010
V5	01001001110010001000001000000100101000001001001010000011000000010
V6	00000010000000000000000000000000000000000001000100000000000010010
V7	00000010000000000000000000000000000000000001010100000000000010000
V8	00000000000000000000000000000000000000000000010001000000000010000
V9	00000010010000000010000000000000000000000001010000000000000110000
V10	00000001000000000000000000000000000000000000000000000000000010000
V11	00000010010000000000000000000000000000000001000100000000000010000
RSP1	01010100011001010110000010001000000000100010010000100000001110010
RSP2	01011110001001010110000101001000000100110010010000100000000100010
RSP3	00000000010000000010000000000000000000000000000000000000001010000
RSP4	11010100011001010110000010001000000000000010011000110000001100011
RSP5	01000100110000010100000100001011001100000100000000100000001000010
RSP6	00000000010000000010000000000000000000000000010001000000000010000
RSP7	11010000111000000101000010000010010010000100100000100100010010000
RSP8	11010101011001010111000110001010010010000100000000100000011000010
RSP9	01001011110011101000000000100000000000010000000100000011001010000
RSP10	01001001111000100100000100001000000000000100100011000000010000100
RSP11	00000000010000000000000000000010000001001000000000000000001110000
RSP12	01001001110001100100000100001000011100000100001001010000010000110

Appendix Table 2: Binary Code with RP4 Primer

Sample	Binary Code
V1	0100000000000000000000000000000001000000000000000001110011000
V2	0010000000000000101000000000000010000000000010000000000000000
V3	0010100000010010000100000000100001001100001100010000000001000
V4	0010000000000010000100000000000000000000000000000000000000000
V5	0010100000010010000000000001000000000000000000000000000001010
V6	0010100000010010000100000000100000000000000000000000000000000
V7	0010000000010010001100000000100001000000000000000000000001000
V8	0011100000000001100100000000000010000000100000000000110101000
V9	0010000000000000010110000000000010000000000010100000000001000
V10	0110100000001101100100000010000001001011000000000000000000010
V11	0000110011011000010001011100011111010000101110000011111001011
RSP1	0100000000000000000000000000000000000000000000000000000000000
RSP2	0100000000000000000000000010000000000000000000000000000000000
RSP3	0000100111000000000000000000000010010110000000000000001000010
RSP4	0000000000000100000000000001000000000000000000000000000000000
RSP5	0100001000000000000000000001000001000000000000001100000100010
RSP6	0000000000000100001000000001000000000000000000000000000000000
RSP7	0000000011000000000000000001000001000000010000000000001000010
RSP8	1000000000000100001000000001000011100010000000000000000000010
RSP9	1000000011000100001000000001000001000000000001000100000000000
RSP10	1000000000000100001000000001000000000000000000000000000000000
RSP11	1000000000100100001000000001000000000000000000000000000000000
RSP12	1000001101000100001000110001000001000000010000000000000000010

Appendix Table 3: Binary Code with RP6 Primer

Sample	Binary Code
V1	01
V2	01
V3	00
V4	00
V5	00
V6	00
V7	00
V8	00
V9	00
V10	10
V11	00
RSP1	00
RSP2	00
RSP3	00
RSP4	00
RSP5	00
RSP6	00
RSP7	00
RSP8	00
RSP9	00
RSP10	00
RSP11	00
RSP12	00

Appendix Table 4: Binary Code with MRJ-1 Primer

Sample	Binary Code
V1	0000000000000100000000000000000000000000000000000000000100000000000100100000000000000000010000000000000000000000000000
V2	0000001000000100100011100000100000010000000010000000000000001000000000010000100000000000100000000100000000000000000000
V3	0000000000000000000000000000000000000000000010000000001000000000000000000000010000000001000000010000000000000000000000
V4	0000001000000000000010000000000000000000000000000010010010001000000000000000100000000000011000000100000000100100000000
V5	0000000000000000000000000000000000000000000000000000010000000000000100000000000000000000000001010000000000001000000000
V6	0000001000101000000000001000000000100000000000000010000010001000000000100001001001000000001000000000001000100000100000
V7	0000001000000000000001000001000000000000000000000010100100000010010000000000001000101000100100000000001000000100000000001
V8	0001000000101000001000000000000000000010110100000000000000001001000000000000000000000000000000001000000000000000010000
V9	0001000000101000001000000000000000000010110100000000000000001001000000000000000000000000000000001000000000000000010000
V10	0000001001101000001010100010010100100000000101000100010010001000010000100000100000100011000001111000010100000010000000
V11	1001001000101000001010000010000000101000100000000000000010000001000000010000100011001000000000100100000000000100000000
RSP1	0001000000000000000000000000000000000000000000001000000001000000000000000000000000000000000010000000000000000000000000
RSP2	0001000010000101000000010000001000000001010000010000011101000100001100010101000001101111011010100000010001111010111100100
RSP3	1100100001000101001000001101001001010010001000000001000100100100000100000010000100000000000000000000010000000000000010000
RSP4	0000100000001000000000000000000000001000000000000000000001001001000000000000000010000000000000000000000000000000000000010
RSP5	0000000000000101001000010000001000000000000000000000000001010001000001000000000010000000000000000000000100000000000000010101
RSP6	0000100000000010000100000000000000000000000000000101000000000000000000000000000000000000000000000000000000000000000000000
RSP7	0000000000000000000000000000000000000000000000000000000000000000000001000000000000000010110000100000000000000000000000000
RSP8	0011010001001000001010000110010010100000100000000010010000001000010000000000000000000001010000000000000000000000000000
RSP9	0000000001101000001010001010010100100000100001000010010010001000100000000000010000000000000000000000000000000001000000
RSP10	0000000000000000010000000000000000000000000000000000010000000000000000000000000000000000010000000000000000000000100000
RSP11	0000000000000000000000000000000000000001000000101000001000000000000000000000000000000000000000000000000000000000000000000000
RSP12	0000000100000100000000000100000000000000000000000000010000000000000000000000000100000000100100000000000001001000000000

Appendix Table 5: Binary Code with MRJ-2 Primer

Sample	Binary Code
V1	0100010010000010000000000010000000000000000000000000010000001100010000001000000000000000000000000000000000000010000000000000000000000000000
V2	0100101101001000000000000010100000000000000000000000000100000010000000000000000000000000000000000000100000100000010000000000000000000100001000
V3	1000010110001001000010010000000000000011000000000100001000000000000000000000000100001000000001000000000000101100000010000000000010001000000000
V4	1010100011000000000011000011100000000000000000000010000000000001000000000000000010001000000000000000000000000000000000010000001001000100000
V5	1010000101100000000000001000000000000000100000000000100000000000000010000000000000000100001000000000000000000000101010000000000000000000000
V6	1100111111011011101011101111010010010101100100010010001010100010000001000100011001000001000111010000001100000010100100001010110010000010000000
V7	0011000000000000000100000000000000000011000000100000000000010001000000000000100000000010000010000000000000000000000001000100000100000000000
V8	0000000010000000000000000010000000000000000000000000000001000000000000100001000000000000010000000001000000000000100000000000000010000000000
V9	0010000010000000000000010001010000000001000000000000000001010000000000000000000000000100000000000000000000010001101000000000000000000000000
V10	0001000010010010000100001101010010100011001000100110101001001000010010001000100010111001000100000000011010100010011000000010000000010000010
V11	0000000010000010000000000000000000000001000000000000000000000000000000000000011101010000001000000100000000000000100100100000100000000001
RSP1	1010000000000100000000100000000010001101101100001000010100010000000000000000000000010000000001010000000001000010001100000100000011100000
RSP2	0000000000000000000000000000000000010000000010001000000000000000010000000000000000000100000000000000000010000000000010000100000000001000001
RSP3	0010010000000000000000100101000010001001101100000000011010000000000010000000001000100000000010010000000010000100101000000100000000100000
RSP4	1010000011000100010000010010100001000100110110000000001010001001100101010000000000000000100001001001000010000100000000010000000000000000000
RSP5	0000000010000000000000000010000000000000000000000100000000000000000110000000100001010000000000100000000000000000000100010000010000000000000
RSP6	0000000010000000000000000000000000000000000000010101010000001001000000000100010000000000000001001000000000000000000000000100101100000000000001
RSP7	1000000010000000010010010010100001000110110110000100001010101000000001010000000001000000001110110001010000000100000100010100100000000010000000
RSP8	1010000010000000000000000000000000000000000000000000000100001000001000000000000000000000000000010110100100100000001000000010000100000000000000
RSP9	1010100011000100010000010101010000000000101001000100001000100000001000111100111000001010000110001100001011000010000101011000100101010001000000
RSP10	1010000011000000010000010000000000000000100000000100000100000000010000000000000000000000000010000100100000000000001001000010000000000000000
RSP11	0000000001000000000001001000000100000011011001000000000101000001000100100000000000000010000000000000000000000000000010000100110000001000100000
RSP12	0000000010100000000000000000000000000000000000000000000000010000001000000000001000001000001001010000000001000000101000000000000000000000110

Appendix Table 6: Binary Code with MRJ-3 Primer

Sample	Binary Code
V1	000000000000000
V2	000000000000000
V3	000000000000000
V4	000000000000000
V5	001000000000000
V6	000000000000000
V7	000000000000000
V8	000000000000000
V9	000000000000000
V10	000000000000000
V11	000000000000000
RSP1	100000000000100
RSP2	110010011111100
RSP3	100000000000000
RSP4	100000000000000
RSP5	100001000010110
RSP6	100000000000000
RSP7	110110100110100
RSP8	110001100011001
RSP9	100000000001100
RSP10	100000000000000
RSP11	000000000000000
RSP12	000000000000000

REFERENCES

Anderson, E. 1949 Introgressive hybridization. Wiley, New York. 109 pp.

Bernatchez, L., Glemet, H., Wilson, C.C., & Danzmann, R.G. 1995 Introgression and fixation of Arctic char (*Salvelinus alpinus*) mitochondrial genome in an allopatric population of brook trout (*Salvelinus fontinalis*). *Canadian J. Fish. Aquat. Sci.* **52**, 179-185.

Best, T.L. 1993 *Tamias ruficaudus*. *Mammalian Species* **452**, 1-7.

Brower, J.V.Z. 1958a Experimental studies of mimicry in some North American butterflies: Part I. The Monarch, *Danaus plexippus*, and Viceroy, *Limenitis archippus archippus*. *Evolution* **12**, 32-47.

Brower, J.V.Z. 1958b Experimental studies of mimicry in some North American butterflies: Part II. *Battus philenor* and *Papilio troilus*, *P. polyxenes* and *P. glaucus*. *Evolution* **12**, 123-136.

Brower, L.P. & Brower, J.V.Z. 1962 The relative abundance of model and mimic butterflies in natural populations of the *Battus philenor* mimicry complex. *Ecology* **43**, 154-158.

Brower, L.P., Brower, J.V.Z., & Corvino, J.M. 1967 Plant poisons in a terrestrial food chain. *Proceedings of the National Academy of Sciences of the United States of America* **57**, 893-898.

Brower, L.P., Ryerson, W.N., Coppinger, L.L., & Glazier, S.C. 1968 Ecological chemistry and the palatability spectrum. *Science* **161**, 1349-1351.

Bull, V., Beltran, M., Jiggins, C.D., McMillan, W.O., Bermingham, E., & Mallet, J. 2006 Polyphyly and gene flow between non-sibling *Heliconius* species. *BMC Biol.* **4**, 11. (doi: 10.1186/1741-7007-4-11)

Chapin, J.P. 1948 Variation and hybridization among the paradise flycatchers of Africa. *Evolution* **2**, 111-126.

Covell, C.V. 1994 Field observations of matings between female *Limenitis archippus* and male *L. arthemis* subspecies (Nymphalidae). *J. Lepid. Soc.* **48**, 199-204.

Coyne, J.A. & Orr, H.A. 2004 Speciation. Sinauer Associates, Sunderland, Massachusetts.

Cunningham, C.W. 1997 Can three incongruence tests predict when data should be combined? *Mol. Biol. Evol.* **14**, 733-740.

Cunningham, C. & Berger, J. 1994 *Bison*. Columbia University Press, New York.

Dasmahapatra, K. K., Silva-Vasquez, A., Chung, J-W., & Mallet, J. 2007 Genetic analysis of a wild-caught hybrid between non-sister *Heliconius* butterfly species. *Biol. Letters*, doi:10.1098/rsbl.2007.0401.

Folmer, O., Black, M., Hoeh, W., Lutz, R., & Vrijenhoek, R. 1994 DNA primers for amplification of mitochondrial cytochrome C oxidase subunit I from diverse metazoan invertebrates. *Mel. Marine Biol. Biot.* **3**, 294-299.

Good, J.M., Hird, S., Reid, N., Demboski, J.R., Steppan, S.J., Martin-Nimss, T.R., & Sullivan, J. 2008 Ancient hybridization and mitochondrial capture between two species of chipmunks. *Molecular Ecol.* **17**, 1313-1327.

Halbert, N.D. & Derr, J.N. 2007 A comprehensive evaluation of cattle introgression into US federal bison herds. *J. Heredity* **98**, 1-12.

Hill, R.I. & Vaca, J.F. 2004 Differential wing strength in Pierella butterflies (Nymphalidae, Satyrinae) supports the deflection hypothesis. *Biotropica* **36,** 362-370.

Hubbs, C.L. 1955 Hybridization between fish species in nature. *Syst. Zool.* **4**, 1-20.

Jiggins, C.D., Mavarez, J., Beltran, J., McMillan, W.O., & Johnston, J.S. 2005 A genetic linkage map of the mimetic butterfly, *Heliconius melpomene*. *Genetics* **171**, 557-570.

Jiggins, C.D., McMillan, W.O., King, P., & Mallet, J. 1997 The maintenance of species differences across a *Heliconius* hybrid zone. *Heredity* **79**, 495-505.

Jiggins, C.D., Naisbit, R.E., Coe, R.L., & Mallet, J. 2001 Reproductive isolation caused by colour pattern mimicry. *Nature* **411**, 302-305.

Kapan, D.D., Flanagan, N.S., Tobler, A., Papa, R., & Reed, R.D. 2006 Localization of Mullerian mimicry genes on a dense linkage map of *Heliconius erato*. *Genetics* **173**, 735-757.

Kronforst, M.R., Young, L.G., Blume, L.M., & Gilbert, L.E. 2006 Multilocus analysis of admixture and introgression among hybridizing *Heliconius* butterflies. *Evolution* **60**, 1254-1268.

Mallet, J. 2005 Hybridization as an invasion of the genome. *Trends Ecol. Evol.* **20**, 229-237.

Mallet, J., Beltran, M., Neukirchen, W., & Linares, M. 2007 Natural hybridization in heliconiine butterflies: the species boundary as a continuum. *BMC Evolutionary Biology* **7**, 28.

Marcus, J.M., Harper, A.L., Hughes, T.M., Johnson, M.J., Maupin, A.B., Polen, A.B., Powell, T.B., Shehan, T.H., Ritland, D.B., & Covell Jr., C.V. ms. Phylogenetics and hybridization in the butterfly genus *Limenitis* (Nymphalidae) and the origins of the aberrant *Limenitis* form *rubidus* (Strecker). Submitted to Molecular Ecology.

Mayr, E. 1963 Animal species and evolution. Harvard University Press, Cambridge, Massachusetts. 797 pp.

Morgan, B. & Marent, T. 2008 Butterfly. DK Publishing, New York, New York. 280 pp.

Mullen, S.P. 2006 Wing pattern evolution and the origins of mimicry among North American admiral butterflies (Nymphalidae: *Limenitis*). *Mol. Phylogen. Evol.* **39**, 747-758.

Naisbit, R.E., Jiggins, C.D., Linares, M., & Mallet, J. 2002 Hybrid sterility, Haldane's rule, and speciation in *Heliconius cydno* and *H. melpomene*. *Genetics* **161**, 1517-1526.

O'Brien, S.J. & Mayr, E. 1991 Bureaucratic mischief: recognizing endangered species and subspecies. *Science* **251**, 1187-1188.

Platt, A.P. 1969 A simple technique for hand-pairing *Limenitis* butterflies (Nymphalidae). *J. Lepid. Soc.* **23**, 109-112.

Platt, A.P. 1975 Monomorphic mimicry in nearctic *Limenitis* butterflies: experimental hybridization of the *L. arthemis astyanax* complex with *L. archippus*. *Evolution* **29**, 120-141.

Platt, A.P. 1983 Evolution of North American admiral butterflies. *Bull. Ent. Soc. Am.* **29**, 10-22.

Platt, A.P. & Brower, L.P. 1968 Mimetic versus disruptive coloration in intergrading populations of *Limenitis arthemis* and *astyanax* butterflies. *Evolution* **22**, 699-718.

Platt, A.P. & Greenfield, J.C. Jr. 1971 Inter-specific hybridization between *Limenitis Arthemis astyanax* and *L. archippus* (Nymphalidae). *J. Lepid. Soc.* **24**, 278-284.

Platt, A.P. & Harrison, S.J. 1994 First record of an heterotic adult female hybrid *Limenitis* (*Basilarchia*) "*rubidus*" (Strecker) (Lepidoptera: Nymphalidae). *Entomol. News* **105**, 33-38.

Platt, A.P., Rawson, G.W., & Balogh, G. 1978 Inter-specific hybridization involving *Limenitis archippus* and its congeneric species (Nymphalidae). *J. Lepid. Soc.* **32**, 289-303.

Porter, A.H. 1989 Genetic evidence for reproductive isolation between hybridizing *Limenitis* butterflies (Lepidoptera: Nymphalidae) in southwestern New Mexico. *Am. Midl. Nat.* **122**, 275-280.

Porter, A. H. 1990 Testing nominal species boundaries using gene flow statistics: the taxonomy of hybridizing admiral butterflies (*Limenitis*: Nymphalidae). *Syst. Zool.* **39**, 131-147.

Poulton, E.B. 1909 Mimicry in butterflies of North America. *Ann. Entomol. Soc. Am.* **2**, 203-242.

Presgraves, D.C. 2002 Patterns of postzygotic isolation in Lepidoptera. *Evolution* **56**, 1168-1183.

Prudic, K.L., Shapiro, A.M. & Clanton, N.S. 2002 Evaluating a putative mimic relationship between two butterflies, *Adelpha bredowii* and *Limenitis lorquini*. *Ecol. Entomol.* **27**, 68-75.

Ramos, I. 2001 "*Battus philenor*" (On-line), Animal Diversity Web. March 25, 2008 http://animaldiversity.ummz.umich.edu/site/accounts/information/Battus_philenor.html.

Remington, C.L. 1968 Suture zones of hybrid interaction between recently-joined biotas. *Evol. Biol.* **2**, 321-428.

Rieseberg, L. H. & Willis, J. H. 2007 Plant speciation. z*Science* **317**, 910-914.

Ritland, D.B. 1990 Localized interspecific hybridization between mimetic *Limenitis* butterflies (Nymphalidae). *J. Lepid. Soc.* **44**, 163-173.

Ritland, D.B. 1991 Revising a classic butterfly mimicry scenario: demonstration of Müllerian mimicry between Florida viceroys (*Limenitis archippus floridensis*) and queen (*Danaus gilippus berenice*). *Evolution* **45**, 918-934.

Ritland, D.B. 1998 Mimicry-related predation on two viceroy butterfly (*Limenitis archippus*) phenotypes. *Am. Midl. Nat.* **140**, 1-20.

Ritland, D.B. & Brower, L.P. 1991 The viceroy butterfly is not a batesian mimic. *Nature* **350**, 497-498.

Schlipalius, D.I., Waldron, J., Carroll, B.J., Collins, P.J., & Ebert, P.R. 2001 A DNA fingerprinting procedure for ultra high-throughput genetic analysis of insects. *Ins. Mol. Biol.* **10**, 579-585.

Sequencher. 2005 Version 4.5. Ann Arbor, MI: Gene Codes Corporation.

Shapiro, A.M. & Biggs, J.D. 1968 A hybrid *Limenitis* from New York. *J. Res. Lepid.* **7**, 149-152.

Sheppard, P.M. & Turner, J.R.G. 1977 The existence of Müllerian mimicry. *Evolution* **31**, 452-453.

Sperling, F.A.H. 1990 Natural hybrids of *Papilio* (Insecta: Lepidoptera): poor taxonomy or interesting evolutionary problem? *Canad. J. Zool.* **68**, 1790-1799.

Stevens, M. 2005 The role of eyespots as anti-predator mechanisms, principally demonstrated in the Lepidoptera. *Biological Reviews* **80**, 573-588.

Strecker, H. 1878 *Lepidoptera, Rhopalocera, and Heteroceras, Indigenous and Exotic, with Descriptions and Colored Illustrations*. Reading, Pennsylvania: Published by the author.

Sutton, D.A. 1992 *Tamias amoenus*. *Mammalian Species* **390**, 1-8.

Swofford, D.L. 1998 PAUP*, Phylogenetic analysis using parsimony (* and other methods). Sunderland, Massachusetts: Sinauer Associates.

Thompson, J.D., Higgins, D.G. & Gibson, T.J. 1994 Clustal W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**, 4673-4680.

Triplehorn, C.A. & Johnson, N.F. 2005 Borror and Delong's Introduction to the Study Of Insects. Thomson Brooks/Cole, Belmont, CA. 864 pp.

Vane-Wright, R.I. 1991 A case of self-deception. *Nature* **350**, 460-461.

Waldron, J., Peace, C.P., Searle, I.R., Furtado, A., Wade, N., Findlay, I., Graham, M. W. & Carroll, B.J. 2002 Randomly Amplified DNA Fingerprinting: A culmination of DNA marker technologies based on arbitrarily-primed PCR amplification. *J. Biomed. Biotech.* **2**, 141-150.

Walsh, B.D. & Riley, C.V. 1869 Imitative butterflies. *Am. Entomol.* **1**, 189-193.

Williams, D.M. 1983 Mate choice in the mallard, pp. 297-310. In Bateson, P. (ed.), Mate choice. Cambridge University Press, New York. 462 pp.

Willmott, K.R. 2003 Cladistic analysis of the Neotropical butterfly genus *Adelpha* (Lepidoptera: Nymphalidae), with comments on the subtribal classification of Limenitidini. *Systematic Entomology* **28**, 279-322.

Wittenberger, J.F. 1983 Tactics of mate choice, pp. 435-448. In Bateson, P. (ed.), Mate choice. Cambridge University Press, New York. 462 pp.