Genetic Diversity of the Shiny Cowbird (*Molothrus bonariensis minimus*): A mtDNA Study on the Variability Within Original and Expanded Range

By

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A thesis submitted in partial fulfillment of the requirements for the degree of

MASTER IN SCIENCES

in

BIOLOGY

UNIVERSITY OF PUERTO RICO

MAYAGÜEZ CAMPUS

2006

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ABSTRACT

The geographic range of the Shiny Cowbird (*Molothrus bonariensis minimus*) was limited to northeastern South America until the twentieth century. However, this range has expanded to the Caribbean and more recently to the United States. It has jeopardized the population of over 200 avian species within its novel expanded range because of its parasitic behavior. The rapid successful expansion of the Shiny Cowbird, if based on a small number of migrants, may have resulted in a loss of genetic diversity. This study examined the Shiny Cowbird's genetic diversity in both its original and expanded ranges by means of mitochondrial DNA. Sequences of 5 genes and the control region reflected a single haplotype in 34 samples in the expanded range, and 2 haplotypes in 3 birds in the original range. Genetic estimates indicated: $\hat{\pi}$ of 0.00043, K of 0.00141, $\hat{H}$ of 0.66667, and F_{ST} of 0.85771. These estimates indicate lack of gene flow and differentiation between the original and expanded range specimens. These results agree with a founder event.

RESUMEN

La región geográfica del tordo lustroso (*Molothrus bonariensis minimus*) estuvo limitado a América del Sur hasta el siglo veinte. Sin embargo, ésta región se ha expandido al Caribe y mas recientemente a los Estados Unidos. Ésta especie ha comprometido las poblaciones de más de 200 otras especies de aves. La rápida sucesión y exitosa expansión del Tordo lustroso, si es basada en un número pequeño de migrantes puede haber limitado la variabilidad genética en ambas áreas. Éste estudio investigó la diversidad genética del tordo lustroso entre especímenes del área original y el área expandida con el ADN mitocondrial. Secuencias de 5 genes y la región control reflejaron un haplotipo en 34 muestras en el área expandida y dos haplotipos en 3 muestras en el área original. Estimados genéticos muestran: $\hat{\pi}$ de 0.00043, K de 0.00141, $\hat{H}$ de 0.66667 y F_{ST} de 0.85771. Estos estimados indican flujo genético limitado y diferenciación entre los especímenes del área original y expandida. Estos resultados concuerdan con un efecto fundador.

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To my hazel-eyed north star...

ACKNOWLEDGMENTS

The completion of this investigation is in part due to the involvement of several persons that throughout the last thirty months have contributed to the crystallization of this investigation. Consequently, I would like to thank all dearly for helping me achieve my goal.

To my family and friends: Marta Rivera Pérez, Adolfo Porrata-Doria Harding, Rivqa Porrata-Doria Rivera, Nannette López, and Sofía Victoria Calmeiro, and Dorado, all of you were my pillars of strength, courage and perseverance.

To the members of my graduate committee I owe high regards: Dr. Juan Carlos Martínez Cruzado, for being an instructive and understanding mentor. Dr. Allen Lewis, for his insightful comments, and above all, for his "I don't know" for when I presented a quirky question. Sr. Ricardo López-Ortiz, for providing a never-ending source of enthusiasm, and for providing all the help I requested.

A special *thank you* to the dedicated personnel in the Department of Biology of the University of Puerto Rico at Mayagüez for their continuous interest and support in my investigation: I especially thank Dr. Fernando Bird-Picó, Dr. Nannette Diffoot, María Mendez, Dr. Adrianne Tossas, Dr. Rafael Montalvo, and Dr. Lucy Bunkley-Williams, Director of the Department of Biology. Also, I thank the College of Arts and Sciences of the University of Puerto Rico for granting my investigation with funds from the Seed Money Program.

To the graduate (Arlin Feliciano, Aury Rodríguez, Kathy Martínez) and undergraduate or "*pichones"* students in the Molecular Genetics Laboratory, with whom, I shared my frustrations and achievements.

To the fellow workers of the Yellow Shouldered Blackbird Recovery Project of the Department of Natural and Environmental Resources; Katsí Ramos, Roseanne Medina, Arnaldo

Falcón, José Camacho and volunteers. In addition, I thank Annette Díaz, from the Puerto Rico Health Department, for helping in the collection of blood samples.

To Robb Brumfield and Donna Dittman of the Louisiana State University Museum of Natural Sciences, Department of Genetic Resources for their assistance in the loan of seven Shiny Cowbirds specimens. Additionally, I thank Dr. Kratter and D. Steadman for providing the skeletal measurements of specimen 35928.

To David Willard, Bird Collection manager of the Field Museum of Natural History, for his assistance in the loan of specimen 389288 in a short notice.

To Olivier Tostain, of EcoBios, for kindly donating two tissue samples of Shiny Cowbirds from French Guiana.

To Barbara MacKinnon de Montes, of the Sian Ka'an Biosphere Reserve in Mexico, and Dr. Reed Bowman, from Archbold Biological Station in Florida, for sharing their observations on the Shiny Cowbird.

To William Post and Catherine Ortega for providing useful information.

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1X	one time the volume
AIV	avian Influenza Virus
bp	base pairs
CDC	Center for Disease Control
cm	Centimeters
cyt b	*Cytochrome* subunit *b*
DNER	Department of Natural and Environmental Resources
FMNH	Filed Museum of Natural History
g	Grams
LSUMNS	Louisiana State University Museum of Natural Sciences
mg	Milligram
mm	Millimeter
mtDNA	mitochondrial deoxyribonucleic acid
NADH2	nicotinamine adenine dinucleotide dehydrogenase subunit 2
NADH6	nicotinamine adenine dinucleotide dehydrogenase subunit 6
PCR	polymerase chain reaction
rpm	revolutions per minute
s	Seconds
SHCO	Shiny Cowbird (*Molothrus bonariensis minimus*)
tRNA	transfer RNA
Ti	Transitions
Tv	Transversions
U	Units
YSBL	Yellow-shouldered Blackbird (*Agelaius xanthomus*)
YWAR	Yellow Warbler (*Dendroica petechia)*
µL	micro liter
µM	micro molar
WNV	West Nile Virus

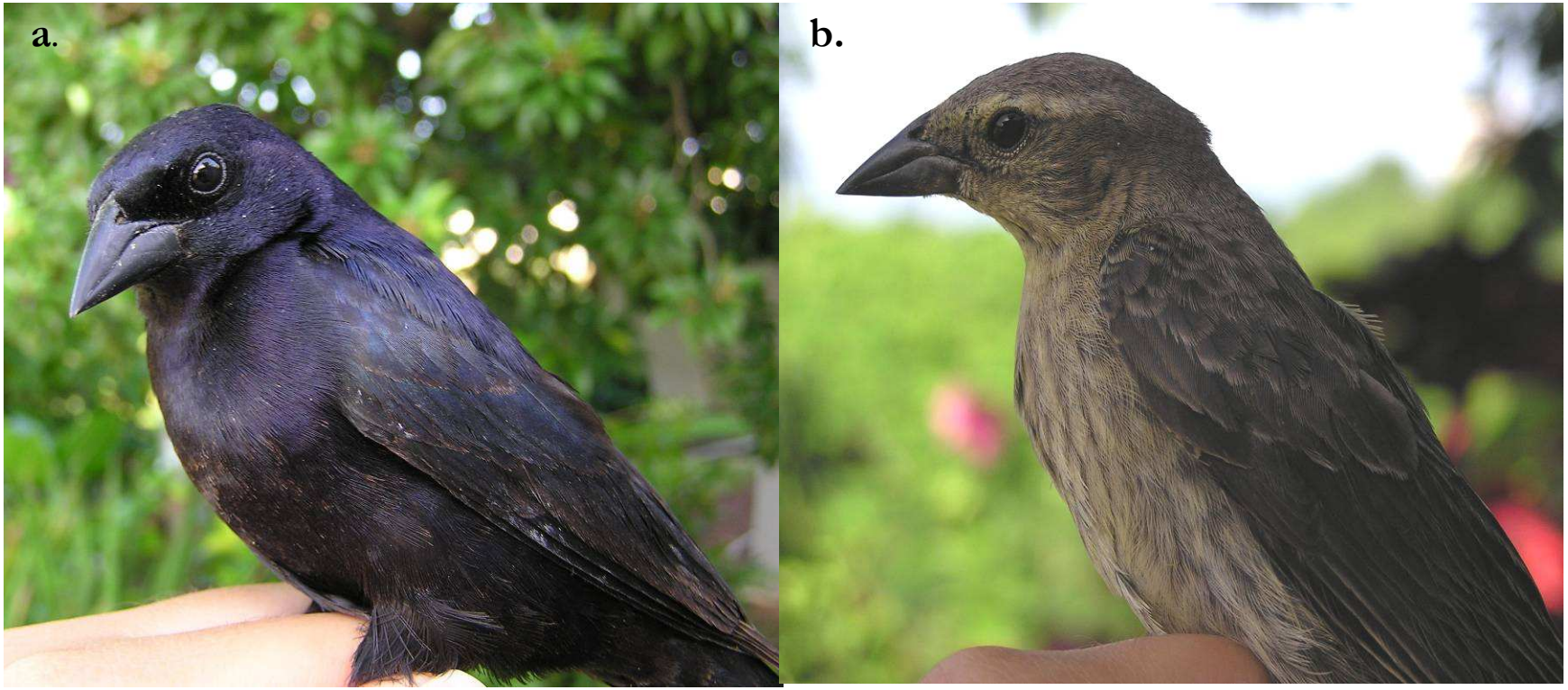
INTRODUCTION

There are seven acknowledged subspecies of the Shiny Cowbird (*Molothrus bonariensis*) (hereafter SHCO) (Blake 1968). These occupy much of the South American continent, and recently one subspecies has been recorded migrating through the Caribbean into the North American continent. The sexually dimorphic subspecies, *M. b. minimus,* is the smallest subspecies with a wingspan of 18 mm (Lowther and Post 1999), and weight of 39 g for males and 32 g for females (Wiley 1986). The adult male (Fig. 1a) presents a glossy purple-black plumage on its head, and dorso; a duller black is present on the wing, rump, and abdomen. Juvenile males are mostly dull brown, but present a mixture of glossy purple-black and brown. Adult females (Fig. 1b) present an ashy-brown crown cut by a lighter colored supercilium. The breast and abdomen have a streaked ashy-brown over a lighter colored brown. The dorso is dull brown and most present a lighter-color on the tips of the feathers. Immature females differ by having dull ashy-brown feathers. SHCO of both sexes and all life stages present black conical bills and legs, and brown eyes.

Native to the northeastern shore of South America, *M. b. minimus* can commonly be found in northern Brazil, French Guiana, Surinam, Guyana, oriental Venezuela, and on the islands of Trinidad and Tobago (Friedman 1929). It has expanded its originally described geographical range, and within the last decades it reached the state of Oklahoma in the USA by 1990, and the province of New Brunswick of Canada in 1993 (Post *et al.* 1993).

As a brood parasite, the range expansion invested by *M. b. minimus* to the Caribbean and North America is an unparalleled event that has affected over 200 avian species within the original and expanded range (Lowther and Post 1999). The manner in which the range expansion occurred is not known. Nonetheless, the range expansion exhibited by the SHCO suggests the aid of anthropogenic factors (Post and Wiley 1977a, Post and Wiley 1977b, Wiley 1985, Wiley 1988).

Figure 1: Male **a.** and female **b**. Shiny Cowbirds (*Molothrus bonariensis minimus*).



There are two main hypotheses that try to explain the geographical range expansion of the SHCO. Wiley (1985) proposed that the SHCO reached Puerto Rico by "island hopping" though the Lesser Antilles. Presumably, the original geographical range of this subspecies may have resulted in occasional wandering to the Lesser Antilles. This hypothesis might explain the sighting of *M. b. minimus* in Vieques by Newton on 1860 without any subsequent sighting until the 1940's. On the other hand, Wiley and Post (1977a) recognize that the male SHCOs attractive song repertoire may have attracted pet traders, and thereafter the accidental or incidental introductions to the West Indies were highly likely. Bond (1976) proposed that the introduction of the SHCO into the Caribbean has two distinct origins one caused by natural factors in the Lesser Antilles, from Grenada to Antigua; and another in the Virgin Islands, the Greater Antilles, and thereafter to North America by an anthropogenic introduction (Post and Wiley 1977 a). Bond (1976) specifies that the SHCO could have spread initially with help of the trade winds to the Lesser Antilles Windward Islands. In addition, the second introduction could have been founded by a single pair of male and female SHCO captured in Barbados and released in St. Croix in 1934 (Bond 1976).

The hypothesis presented for this investigation follows the established *a posteriori* with emphasis on the putative haplotypes in the original and expanded ranges. Thus, in accordance to Wiley's theory of "island hopping", I hypothesized that, if the presence of the SHCO in the Caribbean was due to a casual or accidental migration, the genetic diversity would depend on the number of haplotypes coming from the original area. Meanwhile, corresponding to Bond's theory of two independent introductions, I hypothesized that, if the presence of the SHCO in the Caribbean was due to an anthropogenic introduction of one pair, the SHCOs in the expanded area will only present one haplotype.

The timeline of the range expansion by the SHCO have been well documented in Puerto Rico (Post and Wiley 1977 a, Post *et al.* 1993). However, it is not known with certainty why or how

the range expansion occurred. What fitness consequences, such as are higher adaptability to new environments and higher reproductive output, have been implicated to the SHCO populations in the West Indies and the North American continent has not been investigated.

This investigation explores the effect of range expansion of the SHCO into the Caribbean and North America on the genetic diversity of the subspecies in its new range. It is important to note that with Caribbean I refer to the Greater Antilles, since I was not able to obtain genetic samples of SHCO from the Lesser Antilles. I will try to establish if the SHCO has been historically migrating due to natural factors to the Caribbean as proposed by Wiley. Alternatively, the range expansion is due to human-induced factors as mentioned by Bond. The main objectives of this investigation were to describe the mitochondrial DNA (hereafter mtDNA) diversity of the SHCO in its expanded range, and to analyze comparatively the mtDNA gene sequence composition of SHCOs from the original and the expanded ranges.

LITERATURE REVIEW

I. The cowbirds

The cowbirds compose a New World genus. They are classified under the prolific order Passeriformes; the Icteridae family along other blackbirds, and are grouped into the *Molothrus* genus, which in Latin means "intruder". Many investigations have studied the distinctive intrusive behavior of the cowbirds in the last century (Friedmann 1929, Post and Wiley 1977a, Post and Wiley 1977b, Wiley 1985, Wiley 1988, Cruz *et al.* 1985, Ortega 1998, Cruz *et al.* 2005, López-Ortiz *et al.* 2006).

The intrusive behavior of cowbirds is brood parasitism, in which the cowbird deposits its eggs in a host species nest, so the parasite does not invest in parental care. The host species that accepts the cowbird egg produces less of its own offspring, and hence reduces its reproductive output. Brood parasitism is a breeding strategy used by less than 1% of existing avian species (Mermoz and Ornelas 2003). Not all brood parasites are obligate brood parasites. About twenty-five species of existing birds exhibit obligate brood parasitism (Ortega 1998). However, obligate brood parasitism does not necessarily provide an advantage to the host species. A strong affiliation between host and parasite species is required for parasitism to be successful.

Obligate brood parasitism is thought to have originated independently seven times, as it is observed in seven distinct families or group of species in a family. Families or species that practice this peculiar strategy are honeyguides (Indicatoridae), Old and New World cuckoos (Cuculidae), whydahs and indigobirds (Estrildidae), Parasitic Weaver (Ploceidae), Black-headed Ducks (Anatidae), and the cowbirds (Icteridae) (Gill 2000).

Five species are recognized in the genus *Molothrus*; *M. aeneus*, *M. ater*, *M. bonariensis*, *M. oryzivorus*, and *M. rufoaxillaris* (Ortega 1998). *Agelaioides* (*M.*) *badius*, was recently integrated into a new genus with *A. oreopsar* (Bolivan blackbird) due to mitochondrial DNA evidence that identified these

species as sister taxa (Lowther 2001). *M. oryzyvorus* previously known as *Scaphidura oryzivora* was integrated into the *Molothrus* genus after molecular phylogenetic studies utilizing mtDNA genes showed it to be more closely related to *M. rufoaxillaris* (Johnson and Lanyon 1999, AOU 2000).

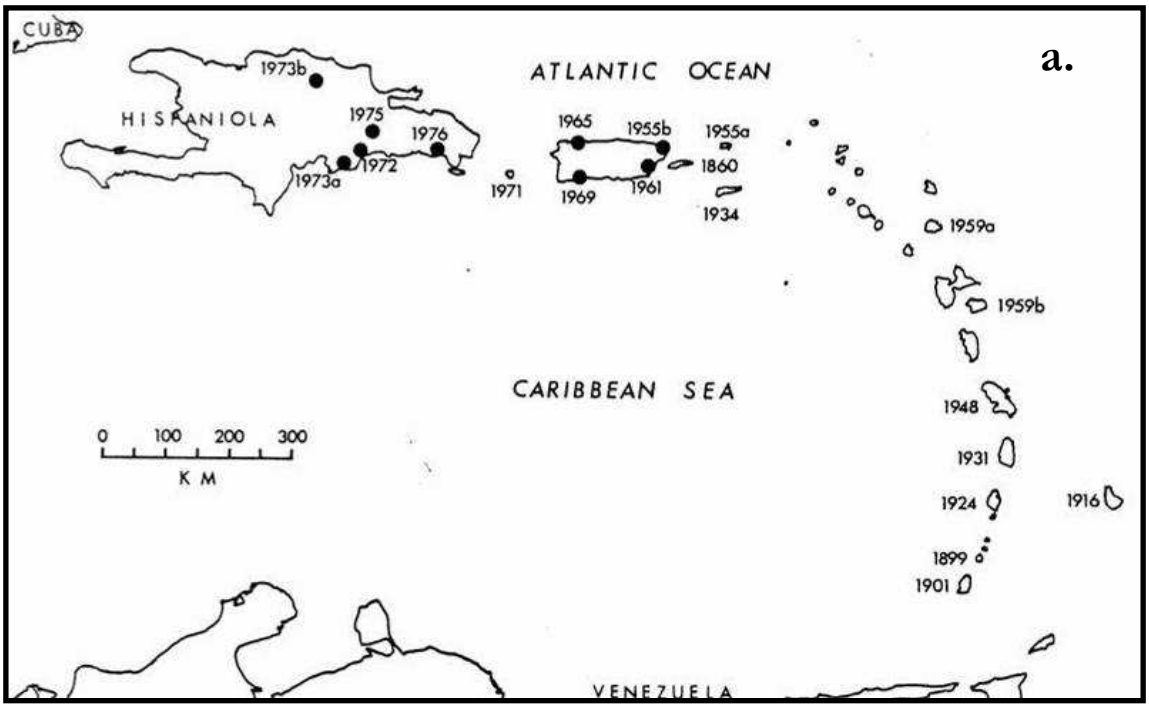
The cowbirds are readily found in disturbed areas such as agricultural lands with patches of trees. They are not known to establish themselves at elevations higher than 2,000 m above sea level (Lowther and Post 1999). Their diet is mainly composed of small arthropods and various grains (*op. cit.*). Even though many aspects of the biology of cowbirds have been investigated since the last century, much information is still missing. For instance, their mating strategy is not known with certainty. Friedmann (1929) indicated that the cowbirds are monogamous. Meanwhile, other investigators have suggested that they might employ a mix of strategies (Lowther and Post 1999).

Ortega (1998) described the following species and subspecies within the Molothrus genus. Most of the *Molothrus* species have been divided into several subspecies. The exception is the South American Screaming Cowbird (*M. rufoaxillaris)*, that has no subspecies described. The Giant Cowbird (*M. oryzivorus*), from Central and South America, has two subspecies: *M. o. oryzivorus* and *M. o. impacifa*. *M. ater*, commonly referred to as the Brown-headed Cowbird, has three acknowledged subspecies: *M. a. ater*, *M. a. artemisiae*, and *M. a. obscurus* are found in North and Central America. *M. aeneus,* or Bronzed Cowbird, is found in southern states of the U. S. A., all of Central America and in Colombia, and it has four subspecies: *M. a. aeneus, M. a. armenti, M. a. assimilis,* and *M. a. loyei.* Finally, the SHCO or *M. bonariensis* is the most diversified with seven subspecies: *M. b. aequatorialis*, *M. b. bonariensis, M. b. cabanisii*, *M. b. minimus, M. b. occidentalis*, *M. b. riparus*, and *M. b. venezuelensis.* The SHCOs occupy most of the South American continent, excepting the high lands in the Andes Mountains in Chile, and continuous wooded areas in the Amazonian forest (Ortega 1998, Lowther and Post 1999).

II. Range expansion of the Shiny Cowbird

All subspecies of the SHCO have been recognized by geographical and morphological differentiation. Three *Molothrus* species or subspecies have modified their original and expanded ranges, I found the identification of specimens through their location less reliable. *M. rufoaxillaris,* for instance, have expanded their range to the northeast of Brazil (Ortega 1998). In addition, *M. ater ater* has been expanding its range, and currently it is found in the southeastern states of the USA (Ortega and Cruz 1992). Furthermore, among the SHCO subspecies, M. *b. cabanisii* is expanding its range into Central America (Ridgely and Gwynne 1989). *M. b. venezuelensis* is presumed to be anthropogenically introduced in Curaçao, because an individual collected had several wing feathers symmetrically clipped (Debrot and Prins 1992). Finally, *M. b. minimus* expanded the native range described by Friedmann (1929) of the northeast shore of South America to the Caribbean (Fig. 2a) and up to North America (Wiley 1985) (Fig. 2b). The SHCO has been documented as west as Oklahoma in the United States in 1990, and as far north as New Brunswick, Canada, by 1993 (Cruz *et al.* 1985, Cruz *et al.* 1989, Cruz *et al.* 2000, Ortega 1998, Lowther and Post 1999). A sighting of a single SHCO male in the Yucatan peninsula in Mexico is documented for May 1996 (Kluza 1998). Even though a confirmation of the identity of the subspecies was not possible, it is most probable that it was a SHCO (*op. cit.*). Additional sightings of SHCOs in the Yucatan peninsula have been made from August 2000 to March 2006 (MacKinnon de Montes pers. comm.).

The augmentation of the range occupied by these species is of much importance, since three of the species that have expanded their range are brood parasites. The SHCOs breeding strategy has compromised the reproductive success of various new host species since it expanded its range (Cruz *et al.* 1985). Perhaps, this is due to the lack of evolutionary strategies by the local island birds against



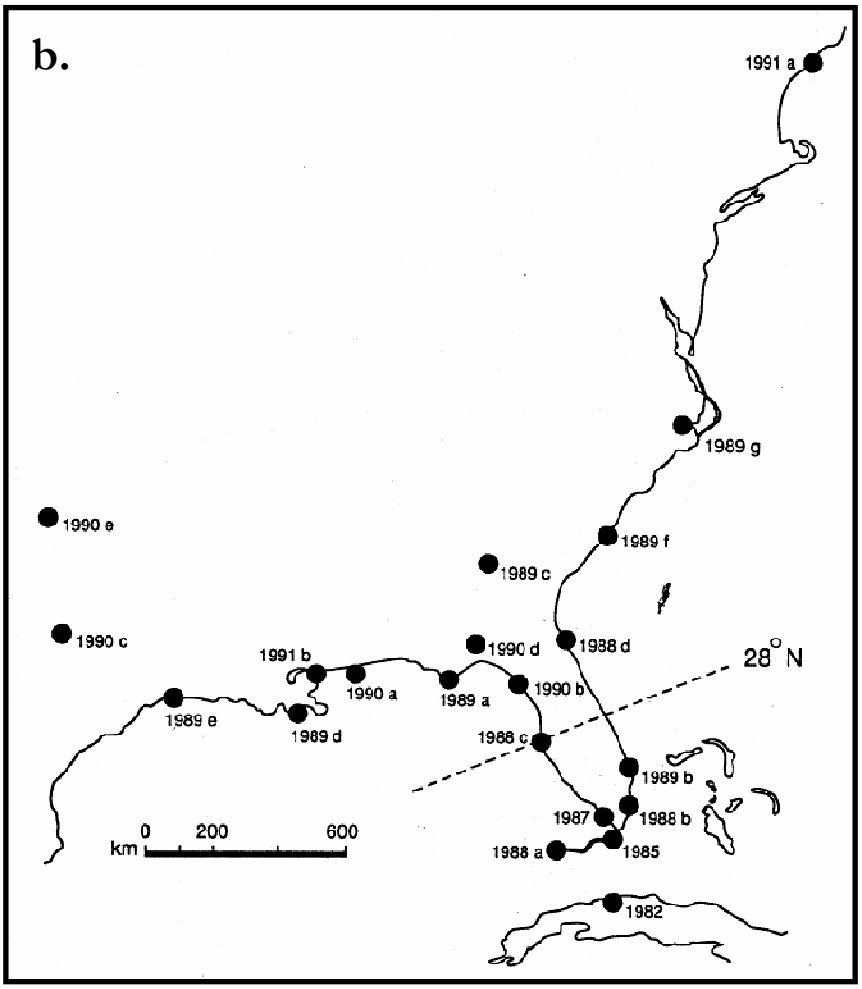


Figure 2: Range expansion of the Shiny Cowbird in the West Indies and the United States. **a**. Range expansion and localities of the sightings of the male or female Shiny Cowbirds through the West Indies from 1860 to 1976 (*source* Post and Wiley 1977a). **b**. Localities of the sightings of the male Shiny Cowbirds in north central Cuba and United States from 1982 to 1991 (*source* Post *et al.* 1993). Sightings are represented with a date and black circles if there are multiple for an island. Letters indicate the earliest records per year.

brood parasites the local island birds(Cruz *et al.* 1989, Woodworth 1997, Nakamura and Cruz 2000, Cruz *et al.* 2005). In the Caribbean, there are no endemic or native brood parasites. In Puerto Rico, however, another brood parasite is present the Pin-tailed whydah (*Vidua macroura*), although its effect has not been studied (Raffaele 2003). The putative anthropogenic introductions of the parasitic SHCO in the Caribbean region have altered at various levels the reproductive success of the local avifauna. For instance, the SHCO is regarded as responsible for the extinction or decline of the House Wren (*Troglodytes aedon*) in Grenada, the Yellow Warbler (*Dendroica petechia*) in Barbados, and the Martinique Oriole (*Icterus bonana*) in the Lesser Antilles (Cruz *et al.* 1985). Meanwhile, in the Greater Antilles, two endemic species of Puerto Rico have been the most affected due to the SHCO parasitizing strategies: the Yellow-shouldered Blackbird (*Agelaius xanthomus*) and the Puerto Rican Vireo (*Vireo latimeri*), (Woodworth 1999, López-Ortiz 2002). The SHCO is now a resident in the Caribbean, and its effect on some endemic species has been well-documented (Arendt and Vargas 1984, Perez-Rivera 1986, Wiley and Post 1976, Wiley and Post 1977a, Wiley and Post 1977b, Wiley 1985, and Wiley 1988).

III. The Shiny Cowbird in Puerto Rico

In Puerto Rico, the SHCO has been found to parasitize at least 17 species (Table 1). However, the SHCOs are known to parasitize approximately 200 species (Friedmann and Kiff 1985, Wiley 1988, Ortega 1998). Complete listings of documented victims and hosts of the SHCO species are available in Lowther and Post (1999), and Ortega (1998). The large number of parasitized species suggests that it follows a host generalist strategy, as has been proposed numerous times (Friedmann 1971, Friedmann *et al.* 1977, Gochfeld 1979, Friedmann and Kiff 1985, Mason 1986, Mason and Rothstein 1986, Mermoz and Fernández 2003).

Puerto Rico has 18 endemic species of birds (Raffaele *et al.* 2003, Oberle 2003, Garrido *et al.* 2005). Of these, three are listed as endangered, the Puerto Rican Nightjar (*Caprimulgus noctitherous*), the Puerto Rican Parrot (*Amazona vittata*), and the Yellow-shouldered Blackbird (*Agelaius xanthomus*) hereafter YSBL. According to the Department of Natural and Environmental Resources (DNER 2004), the U. S. Fish and Wildlife Service (USFWS 1996), and Birdlife International (2004), the first two species are considered as critically endangered while the YSBL is classified as endangered. The YSBL is the only endemic species that has any foreseeable chance of being downgraded to a lesser category of endangerment if implemented management strategies are successful.

Many factors contribute to the endangerment of species, for example, trade, hunting, lack of suitable habitat, hurricanes, and introduced predators (Miner 1996). Another critical factor is the alteration of habitat (Birdlife International 2004, IUCN 2004). Historically, the major cause of avian extinction on islands has been the accidental or intentional arrival of exotic species (King 1985, Blackburn *et al.* 2004). Avian communities on islands often evolve free from competitors, predators, and diseases making these highly vulnerable to the introduction of exotic species (Moors 1985).

Grayce (1957) first noticed a flock of 150 to 175 SHCOs in 1955, even though strong circumstantial evidence suggests that it arrived in Puerto Rico in the 1940's (Post and Wiley 1976, Post and Wiley 1977 b). Since then, the SHCO, because of its obligate brood parasitic nature and its rapid proliferation in Puerto Rico, has been recognized as one of the main factors causing the decline of the YSBLs (Post and Wiley 1976, Post and Wiley 1977a, Post and Wiley 1977b, Post 1981, Cruz *et al.* 1985, Wiley 1985). Extensive efforts have been put into the management of the YSBLs and SHCOs (Wiley *et al.* 1991, López-Ortiz *et al.* 2002, Cruz *et al.* 2005, López-Ortiz *et al.* 2006). The management practices established by the DNER Yellow-shouldered Blackbird Recovery Program, of constantly monitoring and removing of SHCO individuals at any life stage from YSBL nests have contributed to a significant reduction of parasitism in managed areas (Cruz *et al.* 2005, López-Ortiz *et al.* 2006).

Table 1: Species parasitized by the Shiny Cowbird in Puerto Rico

Common Name[1,2] **Scientific Name**	**Measurements of Parasitism (%)**	**References**[3]
Zenaida Dove *Zenaida aurita*	0.4	Pérez-Rivera 1986
Common Ground Dove *Columbina passerina*	2.3	Pérez-Rivera 1986
Puerto Rican Flycatcher[e] *Myiarchus antillarum*	60-85	Ortega 1998, Wiley 1985
Grey Kingbird *Tyrannus dominicensis*	0-8.6	Cruz *et al.* 1985, Pérez-Rivera 1986
Puerto Rican Vireo[e] *Vireo latimeri*	21-83	Nakamura 1995, Woodworth 1997
Black-wiskered Vireo *Vireo altiloquus*	29-82	Nakamura 1995, Wiley 1985
Red-legged Thrush *Turdus plumbeus*	0-4	Cruz *et al.* 1985, Wiley 1985
Northern Mockingbird *Mimus polyglottos*	8-100	Wiley 1985, Cruz *et al.* 1985
Adelaides's Warbler[e] *Dendroica adelaidae*	100	Nakamura 1995
Yellow Warbler *Dendroica petechia*	32-76	Nakamura 1995, Wiley 1988
Black-face Grassquit *Tiaris bicolor*	4.5	Pérez-Rivera 1986
Yellow-Shouldered Blackbird[e] *Agelaius xanthomus*	95	Cruz *et al.* 2005
Greater Antillean Grackle *Quiscalus niger*	0-50	Nakamura 1995, Cruz *et al.* 1985
Puerto Rican Oriole[e] *Icterus portorricensis*	60.7-100	Pérez-Rivera 1986, Wiley 1985
Troupial *Icterus icterus*	0-100	Nakamura 1995, Wiley 1985
Bronzed Mannikin *Lonchura cucullata*	0-17	Cruz *et al.* 1985, Wiley 1985
Nutmeg Mannikin *Lonchura punctulata*	9	Pérez-Rivera 1986

[1]Species in bold are host of the Shiny Cowbird (Lowther and Post 1999).
[2]Common names are those established by AOU.
[3]References are presented as the lowest and the highest measurements of recorded parasitism in Puerto Rico.
[e]Endemic species of Puerto Rico.

IV. Mitochondrial genes as diversity estimators

The mitochondrion, a cellular organelle present in all eukaryote organisms, is specialized in the manufacture of energy. It possesses its own genome that codes for 22 transfer ribonucleic acid (hereafter tRNA) genes, 13 protein-coding genes, two ribosomal RNA (rRNA) genes, and a non-coding region, known as the control region or d-loop. These genes are maternally inherited, and thus do not undergo recombination with other mitochondria (Quinn 1997). Mitochondrial genes are conserved throughout evolution while the non-coding regions have a high substitution rate.

Analyses of mtDNA genes and non-coding regions are used to determine the genetic diversity within species and subspecies (Gibbs 1997). Highly variable molecular markers present in mtDNA are useful tools to measure subpopulation genetic structure and gene flow (Ovenden 1990, Malhi *et al.* 2001). Among commonly used molecular markers, mtDNA is particularly suitable for this study because it provides a more sensitive tool for detecting population subdivision than nuclear genes because of its smaller effective population size (Birky *et al.* 1989). Once an appropriate molecular marker is found, it can be used to estimate the existing variation between subpopulations and within subpopulations.

Estimates that measure genetic diversity are $\hat{H}$, F_{ST}, K, and $\hat{\pi}$. The most common measure of genetic variation in a population is the amount of heterozygosity ($\hat{H}$) at a particular locus (Hedrick 2000). Nei (1987) presented an unbiased estimate of gene diversity, where N is the sample size, and $\hat{p}$ the frequency of the i^{th} allele (Equation 1).

$$\hat{H} = \frac{N}{N-1}\left(1-\sum_{i=1}^{n}\hat{p}_i^2\right) \qquad \textbf{Equation 1}$$

Wright's Fixation Index (F_{ST}) (Equation 2) quantifies variation as $F_{ST} = 1$ indicating complete subpopulation differentiation (different maternal lineages), and $F_{ST} = 0$ indicating that both

subpopulations are really one population (same maternal lineages) (Wright 1951, Weir and Hill 2002).

$$F_{ST} = \frac{H_T - \overline{H}_S}{H_T} \qquad \textbf{Equation 2}$$

Where H_T is the average expected heterozygosity in the total population over loci and H_S is the average expected heterozygosity within subpopulations over loci.

The number of average differences between DNA sequences is also expressed as $\hat{\pi}$. Equation 3 presents the unbiased estimate of the nucleotide diversity expressed by Halliburton (2004), where n is the sample size, *i* and *j* are the alleles, $\hat{p}_i$, $_j$ are the proportions of the alleles and $\hat{\pi}_{ij}$ is the number of differences between the alleles. It is estimated by calculating the average number of nucleotide differences observed among all possible pairs of several sequences (Nei and Li 1979).

$$\hat{\pi} = \frac{n}{n-1}\sum_{i=1}^{k}\sum_{j=1}^{k}\hat{p}_i\hat{p}_j\hat{\pi}_{ij} \qquad \textbf{Equation 3}$$

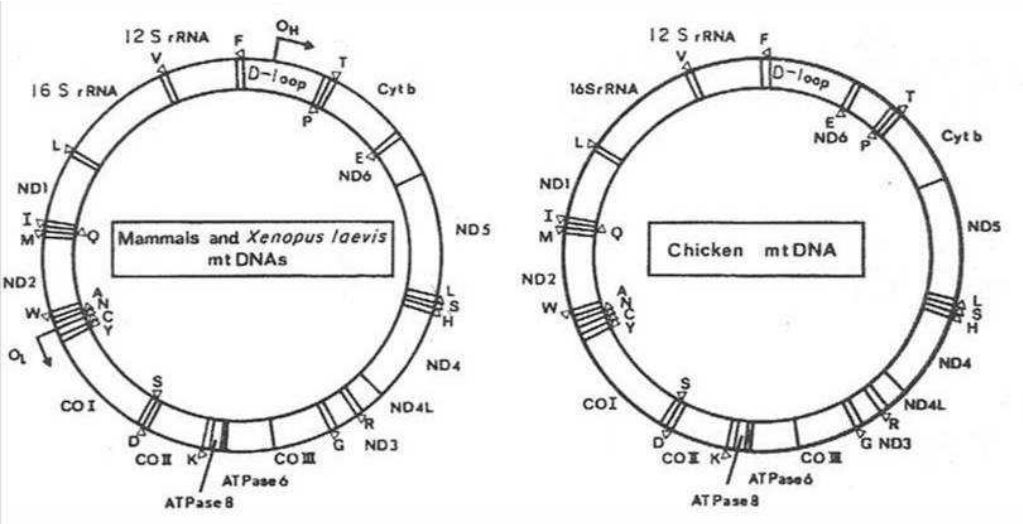
K expresses the number of segregating sites adjusted to sample size. Equation 4 expresses how to obtain the number of segregating sites as presented by Watterson (1975), were *S* is the number of polymorphic sites, and *n* is the sample size.

$$K = \frac{S}{\sum_{i=1}^{n-1}(1/i)} \qquad \textbf{Equation 4}$$

V. Mitochondrial genes and region of interest

The molecular organization of vertebrate mtDNAs has suffered little change throughout evolution (Anderson *et al.* 1981, Bibb *et al.* 1981, Arnason and Gullberg 1993, Xu *et al.* 1996a, Xu *et al.* 1996b, Xu and Arnason 1997, Kumazawa *et al.* 1998, Nilsson *et al.* 2003). Control region sequence studies in vertebrates have usually shown central conserved sequences flanked by regions that are more variable. For example, in humans, two hypervariable regions have been identified within the control region, one on each side of a low mutation rate region (Greenberg *et al.* 1983, Vigilant *et al.* 1989). Nonetheless, bird mtDNAs are distinctive in the order of the genes that lie next to the right end of the control region (Desjardins and Morais 1990) (Fig. 3). Specifically, the $tRNA^{Glu}$ and *NADH6* genes border the right end of the control region instead of the $tRNA^{Pro}$, $tRNA^{Thr}$ and *cytochrome b* (hereafter *cyt b*) genes. Figure 3 shows the noted differences.

Figure 3: MtDNA genome organization in mammals and the African clawed frog (*Xenopus laevis*) compared to that of white Leghorn chicken (*Gallus gallus domesticus*). Protein and tRNA genes shown outside the circle are H-strand encoded with counter-clockwise polarity. The *NADH6* gene (ND6) shown inside the circle is L-strand encoded with clockwise polarity. *Source*: Desjardins and Morais 1990.



Few population studies have been performed with mtDNA on birds to identify hypervariable regions within the control region. A population study was performed on the Black-

backed Oriole (*Icterus abeillei*) in which 344 bp including positions 104 to 447 starting from the first base of the $tRNA^{Glu}$ were sequenced in nine birds from various Mexican regions (Kondo *et al.* 2004). Three variable sites were found, and thus the magnitude of the variation within the region was similar to a hypervariable region in humans. In addition, a study performed by Growth (2000 unpubl.) of finches including four species of *Loxia* and one of *Carduelis*. For which, the region spanning the *cyt b*, *NADH6*, control region, and 12S ribosomal RNA genes was sequenced, unexpectedly showed that variation within the *NADH6* gene (3.7%) was higher than in the control region (1.6%), and that variation within the control region was not higher than in the *cyt b* gene (1.6%) (GenBank accession numbers AF171652-AF171664). Therefore, it was not assumed in this study that the control region would be the most informative mtDNA region for this study. The genetic diversity of the SHCO was assessed employing several genes and the control region.

A small number of investigations have mtDNA and the species of interest for molecular research. Freeman and Zink (1995) investigated the phylogeny of 47 species of blackbirds based on the mtDNA variation in restriction sites. They concluded that the phylogenetic signal was stronger in sequence data than in restriction site data. However, the lack of information gained through restriction site data has been reinforced with other investigation that have dealt with establishing phylogenies of the New World blackbirds and brood parasitism origin with nucleotide sequences. Some of these investigations have generated sequences that have been published in GenBank (www.ncbi.nlm.nih.gov/) and served as source of selection of the genes to be used in this study. Kocher *et al.* (1989) presented universal primers for vertebrates in three regions of the mtDNA: 12 S rRNA, control region, and *cyt b*. These primers were used successfully in over 100 vertebrate species, including fishes, amphibians, birds, and mammals, and amplifies a fragment of 307 bp. Lanyon (1992) added oligomers B3 and B4 that can be used to amplify a 726 bp fragment within the

cyt b gene. Nucleotide sequences for the *cyt b* and *NADH2* genes were available for this subspecies thanks to Lanyon (1999) and other collaborators.

Lack of oligomers to sequence other genes potentially more informative than *cyt b* motivated Sorenson *et al.* (1999) to publish 86 sets of oligomers for avian and other vertebrate mtDNA. In addition, Sorenson and Payne (2001) provided two sequences of *M. b. occidentalis* including *NADH6*, $tRNA^{Glu}$ and the control regions (GenBank: AF407133); and the $tRNA^{Phe}$ and 12S rRNA genes (GenBank: 407090). Sequence AF407133, of 1,090 bp, contained a portion of the *NADH6* gene and of the control region, as well as the complete sequence of the $tRNA^{Glu}$ gene that lies between them. Sequence AF407090, of 1,013 bp, contains partial sequences of the $tRNA^{Phe}$ and 12S rRNA genes. A third sequence (AF171663) from a different genus, *Loxia curvirostra stricklandi,* of 4,248 bp, contained the following genes or DNA regions: $tRNA^{Thr}$, $tRNA^{Pro}$, *NADH6*, $tRNA^{Glu}$, $tRNA^{Phe}$, control region, 12 S rRNA, and $tRNA^{Val}$. This sequence was used in this study along with the two former sequences to design three sets of primers to amplify the remainder of the control region in *M. b. minimus* together with the entire *NADH6*, $tRNA^{Glu}$ and $tRNA^{Phe}$ genes, plus part of the $tRNA^{Pro}$ and the 12 S RNA genes. This investigation amplified and sequenced the control region, and the *cyt b*, *NADH2*, *NADH6*, $tRNA^{Glu}$, $tRNA^{Phe}$ genes.

MATERIALS AND METHODS

I. Samples

Genetic samples from 38 SHCOs, ranging from the continental USA in the states of Louisiana and Florida; Puerto Rico, Brazil, French Guiana, and Trinidad and Tobago were obtained for this investigation (Table 2 and Fig. 4). These included four from its original range and thirty-four from its expanded range. Blood samples from adult SHCOs were collected from August 2004 to July of 2005, and the collection of eggs were held from August of 2004 to June of 2005. Frozen tissues from seven specimens were obtained from Louisiana State University Museum of Natural Science (LSUMNS), Collection of Genetic Resources Lab, and a sample was acquired from the Field Museum of Natural History (FMNH), Bird Collection. In addition, Olivier Tostain from French Guiana kindly donated samples from two preserved specimens.

A. Blood samples from adult Shiny Cowbirds

I. Capture

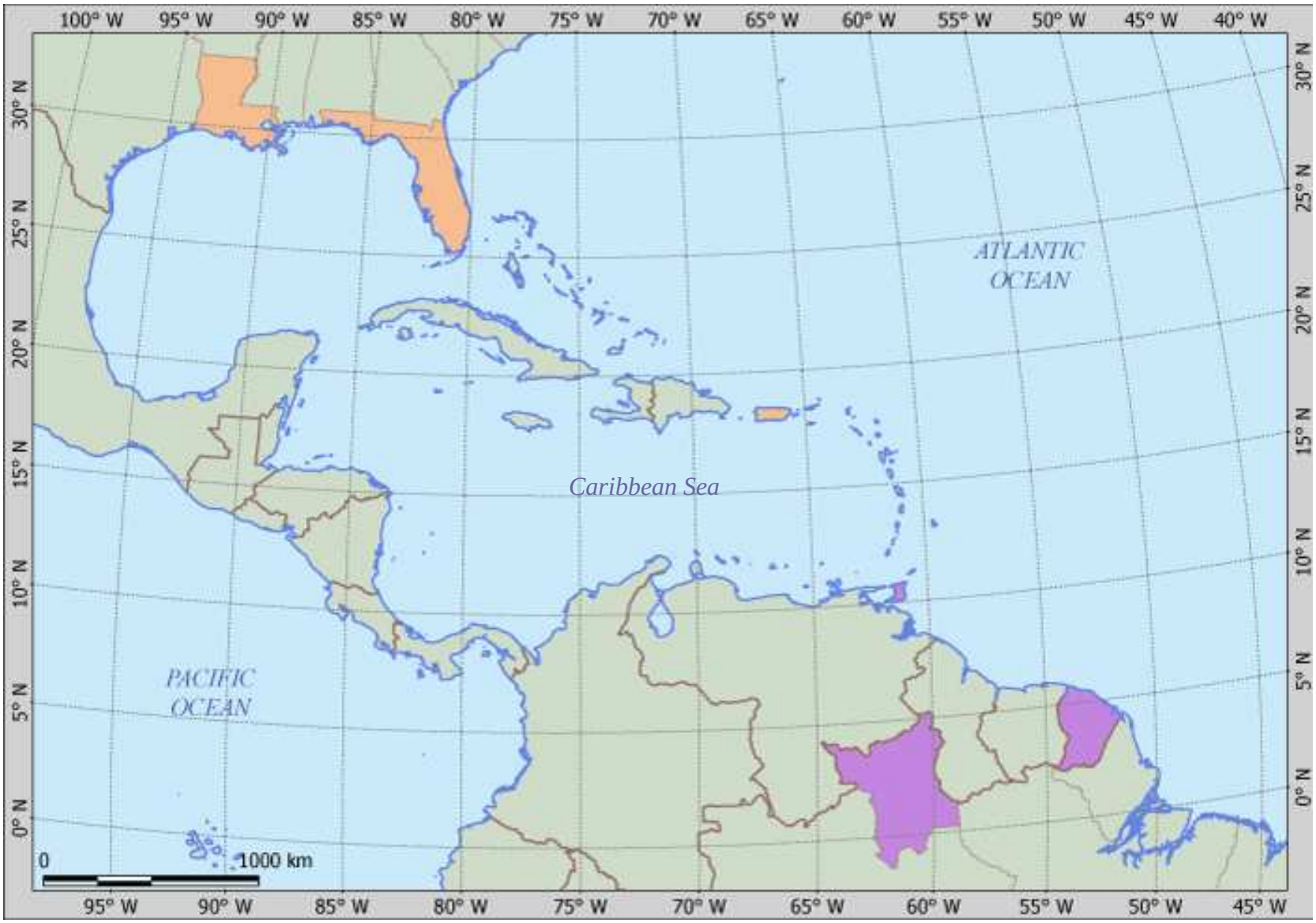
SHCOs were collected in Puerto Rico in cooperation with the Puerto Rico Department of Natural and Environmental Resources (DNER) Yellow-shouldered Blackbird Recovery Project (03-EPE-022). Samples were collected in 1.5 m^3 cages of galvanized wire, which included a wire excluder. The excluder served by filtering birds larger than SHCOs. These cages were placed in Estación Experimental Agrícola in the municipality of Lajas, and Vaquería Ortiz of Boquerón in the municipality of Cabo Rojo with plenty of food and water. In addition, 1 to 5 male cowbirds were placed in the cage as decoy.

Table 2: Information of the eleven sampled populations of Shiny Cowbirds (*Molothrus bonariensis minimus*).

Range	Country	State or Municipality	Locality	Year of collection	Sample ID (*n*=38)	Life stage/ Sex[1]	Type[2]
Expanded	Puerto Rico	Cabo Rojo	Road 301	1987	11476 11479 11478, 11480	J/M A/F J/F	T[a]
			Vaquería Ortiz	2005	Bo7-8	J/M	B
		Fajardo	Las Croabas	2005	Fa3, 4, 5 Fa1, 2	A/M A/F	B
		Hatillo	Vaquería Amador	2005	Ha1, 3, 5 Ha2 Ha4, 6, 7	J/M A/M A/F	B
		Lajas	Agricultural Experimental Station	2005	La3-8	J/M	B
			Pitahaya	2004	D10, N9, N15, 38, 51	E/U	T
		Maricao	Maricao State Forest	2004	Ma1	A/F	B
		Vieques	Mosquito Bay	2005	Mo1a-b	E/U	T
	USA	Florida, Alachua Co.	Micanopy Historic District	2000	49210	U/M	T[a]
		Louisiana, Cameron Parish Co.	Ca 5 mi. east Cameron	1999	28864	J/M	T[a]
Original	Brazil	Roraima	Fazenda Santa Cecilia, east Rio Branco bank	1992	389288	A/F	T[b]
	French Guiana	Kourou	Pointe des Roches	2006	475m 475f	U/M U/F	T[c]
	Trinidad and Tobago	Caroni	Caroni rice fields	2002	35928	A/F	T[a]

[1]A=Adult, E=Embryo, J=Juvenile, M=Male, F=Female, U=Unknown; [2]T=Tissue, B=Blood
[a]Samples provided by LSUMNS. [b]Sample provided by FMNH. [c]Samples provided by Olivier Tostain.

Figure 4: Location of the sampled populations of Shiny Cowbirds (*Molothrus bonariensis minimus*) in the expanded (orange) and original (purple) range.



II. Collection of blood samples

Blood samples from live adult birds were obtained from the jugular vein. The blood (200 to 350 µL) were extracted and placed in a sterile 1.5 mL microtube with 1 mL lysis buffer as described by Longmire (1992).

III. DNA extraction

For each individual sample, 250 µL of blood in Longmire buffer was placed in a sterile 1.5 mL microtube, and 6.25 µL of proteinase K (20 mg/mL) were added. The mix was incubated overnight rotating at 37°C. The next day, 1X volume of phenol-chloroform-isoamyl alcohol (25:24:1) (Fisher, BP17521) was added, and the mix was left rotating in the incubator at 37°C for 1 h. Then, the samples were centrifuged at 14,000 rpm for 2 min. The aqueous upper phase was transferred to a sterile 1.5 mL microtube and the organic phase was discarded. 1X volume of chloroform was added, and the mix was vortexed for 5 min and centrifuged at 14,000 rpm for 2 min. The supernatant was transferred to a new 1.5 mL microtube and 0.1X volume of 3M sodium acetate and 1 µL of glycogen (20 mg/mL) was added, followed by the addition of ice-cold 3X volume of ethyl alcohol 100% and centrifugation at 14,000 rpm for 5 min. The supernatant was discarded, as a pellet was visible at the bottom of the microtube. The pellet was rinsed twice with ice-cold 70% ethyl alcohol, and left to air-dry. The dried pellet was resuspended with 200 µL of TE (10mM Tris-HCL ph7.5: 1mM Na_2EDTA). The product was stored at -20°C.

B. Tissue samples from Shiny Cowbird eggs

I. Capture

Eggs were collected in sector Pitahaya in the municipality of Lajas on Yellow-shouldered Blackbirds nests and in Mosquito bay in the municipality of Vieques on Yellow Warbler nests. The identification of SHCO eggs was confirmed with coloring pattern and size (Appendix I and II).

II. DNA extraction

The eggs were carefully observed, weighed, and cleansed for the extraction of DNA of each embryo. The embryos were processed as directed by QIAGEN's QIAamp® DNA Mini Kit Tissue Protocol.

C. Tissue samples from frozen or preserved SHCOs

I. LSUMNS sample permits

DNER authorized permit number 05-IC-056 for the importation of samples of seven LSUMNS specimens to Puerto Rico, and exportation of seven SHCO fledglings to LSUMNS as part of a mutual benefit requirement. USFWS cleared the exportation of LSUMNS samples with permit number MB756307-0.

Specimen 35928, from Trinidad will be treated as an unknown subspecies for *M. bonariensis* species. A. Kratter identified this specimen after preservation as *M. b. minimus*, but relied mostly on location of the collection of the specimen rather than the measurements. The measurements of the specimen were; wing ch. (unflattened) 93 mm, tail 72 mm, and ex. culmen 16.5 mm. Samples arrived frozen.

II. French Guiana sample permits

Dr. Céline Dupuy, veterinary inspector in French Guiana, awarded a sanitary certification (SA0600580) for both *M. b.minimus* male and female specimens as free of avian influenza on August 28, of 2006. Dr. Arnaud Martrenchar, Departmental Director of the Veterinary Services in French Guiana authorized transportation of the samples from Cayenne, French Guiana to Mayagüez, Puerto Rico. Samples arrived in 90% EtOH.

III. FMNH sample permits

Dr. Dave Willard authorized the tissue sample grant on September 2006 for specimen 389288 from Roraima, Brazil. Samples arrived in 90% EtOH.

IV. DNA extraction

DNA purification from frozen tissue were obtained by cutting 25 mg of muscle tissue, and processing them as directed by QIAGEN's QIAamp® DNA Mini Kit Tissue Protocol.

D. Gel electrophoresis

The visualization of electrophoretic bands obtained from DNA extractions was always consistent with a size of at least 17 kb, and thus of good enough quality for PCR amplification. The molecular marker used was λ DNA-*Hin*d III Digest (New England Biolabs). Electrophoretic gel concentrations were of 0.8% agarose.

II. Molecular research

This investigation took place in the Molecular Genetics Laboratory, Biology Department of the University of Puerto Rico, Mayagüez Campus. The methodologies stipulated were: PCR amplification, purification of the amplified fragments, sequencing the amplified fragments, and analysis of the sequences. These protocols are described in detail below:

A. PCR amplification

I. Oligonucleotides

Table 3 describes the specifications of the oligonucleotides generated and used in this investigation.

II. Reactions

Total PCR of 50 µL required: 1 µL of purified DNA, 1 µL of 20 µM each of a L and a H oligonucleotide, 8 µL dNTPs (2.5 mM), 3 µL $MgCl_2$ (25 mM), 5 µL 10X PCR buffer (Sigma Chemical Co., D8312), 1 µL of BSA (10 µg/µL), 27 or 29.4 µL distilled and de-ionized water, and 3 µL of RED *Taq*™ genomic DNA polymerase (1 U/µL, Sigma Chemical Co.) or 0.6 µL of Taq DNA Polymerase (5 U/µL, Promega).

III. Cycles

Amplification cycles took place in an Eppendorf® Mastercycler. The amplification started with the denaturalization of the double stranded DNA by heating the reaction to 94°C for 2 min 30 s. This was followed by a minimum of 32 and a maximum of 40 cycles of denaturalization at 94°C for 30 s, oligonucleotide annealing to the single-stranded DNA at a temperature between

Table 3: Description and specification of the oligonucleotides used to amplify the *NADH6*, *NADH2*, $tRNA^{Glu}$, $tRNA^{Phe}$, and *cyt b* genes, and the control region in the Shiny Cowbird mtDNA. L and H refers to the light and heavy strands that are extended from the respective primers.

Fragment	Oligos	Sequence	Number of bases	Annealing temperature (°C)[1]	Fragment size (bp)
NADH6 and RNA^{Glu}	LPro	caaaccttcatcaccacctcc	21	56	670
	H562	atgatctaggcgttcctgtag	21		
Control region and $tRNA^{Phe}$	L630	gttttcttctttatttccagg	21	53	690
	H304	atggacatgtcaagaggaag	20		
	L1230	actctcattacctcggcatac	21	58	1,290
	H1954	ctacactggagcgcggatac	20		
NADH2	LND2	gttcaactccttcccctgctaatg	24	60	1,060
	HND2	cttccggaagtttcggaatttgttc	25		
cyt b	LB1[a]	ccatccaacatctcagcatgatgaaa	26	56	307
	HB2[a]	gcccctcagaatgatatttggcccca	26		
	LB3[b]	atctgcatctacctacacatcgg	23	58	726
	HB4[b]	gatgaatgggtgttctactggttg	24		

[1]Anneling temperature used in PCRs.
[a]B1 and B2 oligomers, modified from Kocher *et al.* (1989), these are referred to as L14841 and H15149.
[b]B3 and B4 oligomers, referred by Lanyon (1992) as L15042 and H15767.

53°C and 60°C for 1 min (Table 3), and polymerase-mediated single-stranded DNA extension at 72°C for 1 min 10 s. PCR amplification was finalized with a 10 min period of DNA extension at 72°C. PCR products were stored at -20°C.

IV. Gel electrophoresis

All amplification fragments were confirmed with the visualization of an electrophoretic band of the desired molecular weight (Table 3). The molecular marker used was ΦX 174-DNA *Hae* III Digest (New England Biolabs). Electrophoretic gels fluctuated in concentration between 1.6% and 3% agarose.

B. PCR purifications

Amplifications that yielded desired results were purified as directed by Roche Applied Diagnostics® High Pure PCR Product Purification Kit (1 732 676). All purified fragments were confirmed of the desired molecular weight, and quantified in concentration utilizing 0.5 μg of ΦX 174-DNA *Hae* III Digest as the molecular marker used in 1.6% or 3% agarose electrophoretic gels.

C. Sequencing

The purified PCR products were sent to the Molecular Resources Facility of the University of Medicine and Dentistry of New Jersey <http://njms.umdnj.edu/research/mfrweb/index.htm> for sequencing. The same primers used for amplification, diluted to a 1 μM concentration with TE were used for sequencing.

D. DNA analysis

Computer programs, such as, Omiga™ (GCG, version 2.0, Omiga 1999), Chromas® (Technelysium, version 1.62 ©, 2000), and BioEdit Sequence Alignment Editor © (version 7.0.5.3, Hall 1999), MEGA (version 3.1, Kumar *et al.* 2004), were used to align, edit, and analyze sample sequences.

RESULTS

Great difficulty was found in the permit acquisition of new genetic samples from other countries. The process of permit acquisition was long and complex. For instance, the acquisition of the samples from seven specimens from LSUMNS required seven months to conclude. Investigators and governmental officers from the original range were extremely hard to access and were not at disposition to provide help. As a result, the genetic samples acquired for this investigation outside of Puerto Rico were limited to those available in museums or from donations.

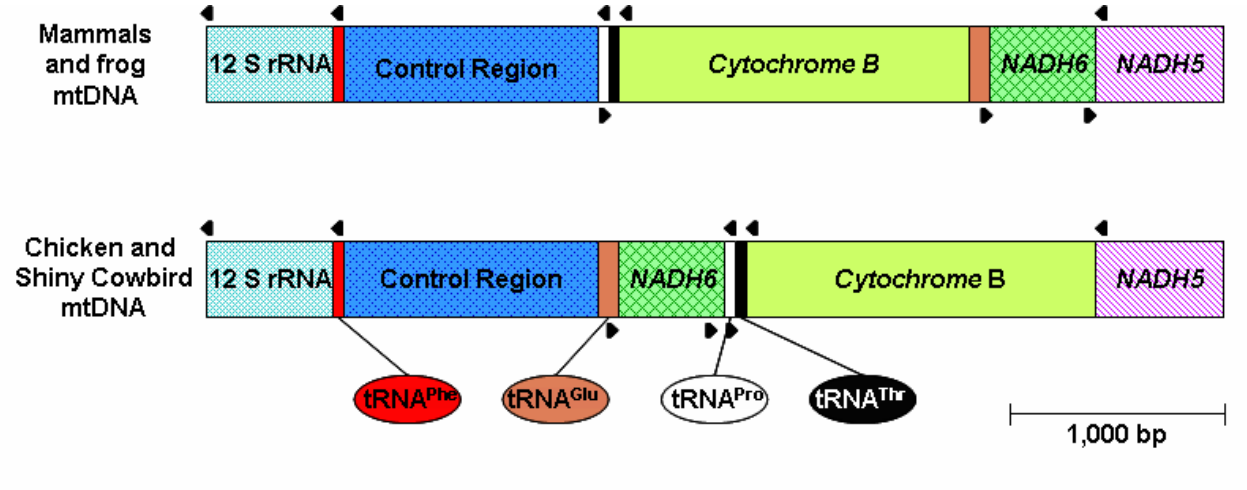
One complication was the need to screen for the latent and recurrent highly pathogenic mutant strains of the Avian Influenza Virus (AIV), particularly the H5N1 strain. AIV has provoked a worldwide implementation of strategies on the prevention, maintenance, and spread of mutant and highly pathogenic strains to wild and domesticated avian species as well as to humans. Non-virulent stains of AIV have been reported sporadically in Puerto Rico (CDC 2005). However, due to Puerto Rico's strategic geographical location, virulent strains of AIV can arrive with migrating birds or through the importation of unscreened products as has been reported in other countries (Hulse-Post *et al.* 2005, Chen *et al.* 2006).

Another virus that has altered guidelines on the control of birds is the West Nile Virus (WNV). Several cases of the WNV have been reported recently in the USA. Occurrences of the WNV were confirmed by the necropsy results of encephalitis presented by many icterids species (CDC 2006). WNV was reported in the migrant Black and White Warbler (*Mniotilta varia*) in the municipality of Ceiba, Puerto Rico (Dupis *et al.* 2003).

I. Molecular organization of the mitochondrial genes in the Shiny Cowbird

This investigation agrees with the mtDNA gene order presented by Desjardins and Morais (1990), as all samples of SHCOs mtDNA amplified accordingly (Fig 5).

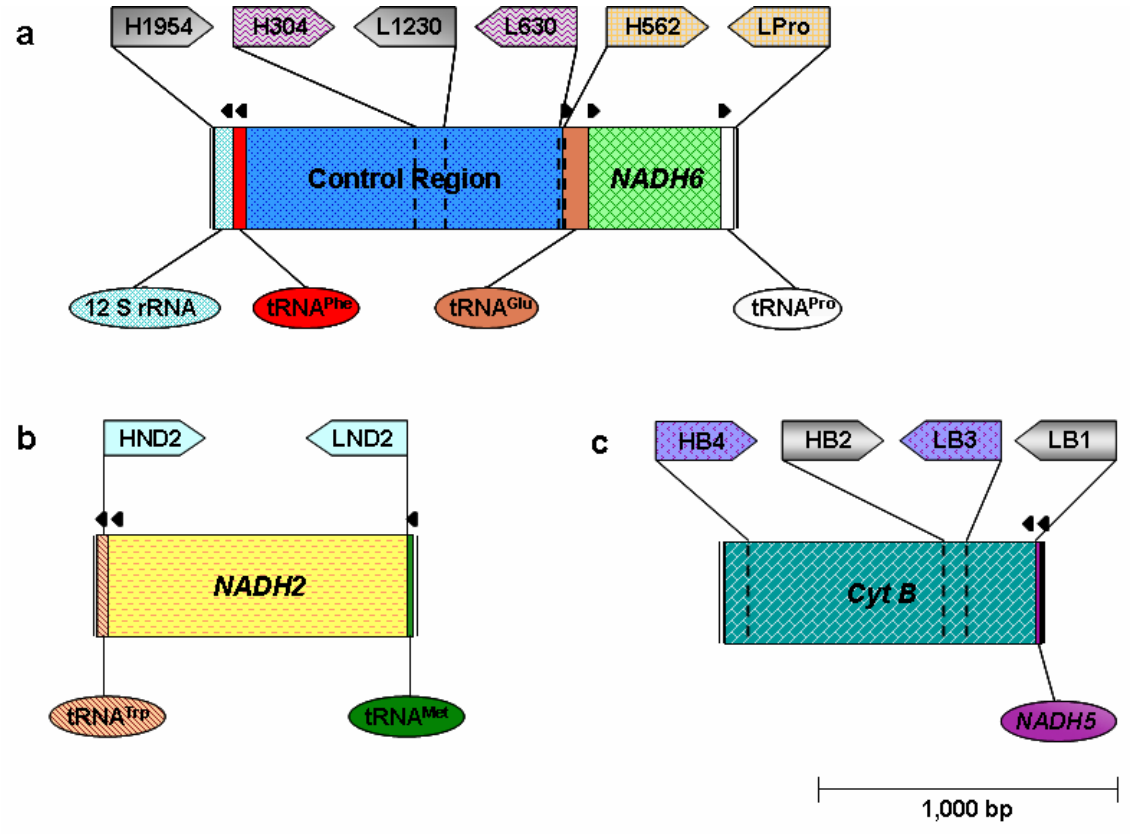
Figure 5: Comparison of the molecular organization of the mitochondrial genes in mammals and the African clawed frog (*Xenopus laevis)* versus white Leghorn chicken (*Gallus gallus domesticus*) and the Shiny Cowbird (*Molothrus bonariensis minimus*). Black arrows indicate the direction of transcription of the genes. Molecular organization is presented as described by Desjardins and Morais (1990).



II. Amplified regions

Amplified mtDNA regions of the SHCO included a complete sequence of *NADH6*, $tRNA^{Glu}$, and $tRNA^{Phe}$ gene regions and most of the control region, and the *cyt b*, *NADH2* genes (Fig 6 a-c). Specifically, the control region was not amplified completely due to a C-run located between oligonucleotides L630 and H562, which could not be amplified.

Figure 6: Amplified fragments of the Shiny Cowbird (*Molothrus bonariensis minimus*) mtDNA. Oligonucleotides are presented in block arrows. Black arrows indicate the direction of transcription of the genes. Dashed-lines specify amplification origin. Double-lines at the end of the fragments indicate not amplified completely. **a**. From right to left, LPro and H562 directed amplification of a fragment that began in the last 25 bases of the $tRNA^{Pro}$ gene, and ended at position 632 in the $tRNA^{Glu}$ gene. The second oligomer set (L630 and H304) generated a fragment that began at the base 669 in the control region, and ended at position 1348. The last oligomer set (L1230 and H1954) produced a fragment that began at base 1279 in the control region and terminated within the first fifty bases of the ribosome subunit 12 S gene. **b**. Amplification of the *NADH2* gene commenced on the 32nd nucleotide base of the gene and concluded on base 1032. **c**. Amplification of the *cyt b* gene was done with LB1 and HB2; these originated a fragment that started in the 99th base pair within the *cyt b* gene and finished within the first 430 bases of the *cyt b* gene. A second set of oligomers, LB3 and HB4, overlapped the amplification of the LB1 and LB2 oligomers, starting around the 300th bp and finishing at base 1,023 of the gene.



III. Sequences

Expanded sequences are those obtained from individuals of Puerto Rico and the USA. Original sequences are from Brazil (B), French Guiana (FG), and Trinidad (T). A period in a column indicates that the same nucleotide appears in that position as at the top of the column.

Figure 7: Shiny Cowbird control region nucleotide sequence of expanded and original range. According sequence AF407133 of *M. bonariensis occidentalis,* this sequence commences at the 58th base pair in the control region, and culminates at the end of the region according to sequence AF171663 of *Loxia curvirostra stricklandi.* A tilde, at position 1,111, indicates a deletion in the sequence of the Trinidad specimen.

```
                    10         20         30         40         50         60
                    |....|....|....|....|....|....|....|....|....|....|....|....|
Expanded            GTATGTATAGAATGCATTACACTCTTTGCCCCATCAGACAGCCCATGTAATGTAGGATAA 60
Original-B          ............................................................ 60
Original-FG         ............................................................ 60
Original-T          ..........................................T....A..........  60

                    70         80         90         100        110        120
                    |....|....|....|....|....|....|....|....|....|....|....|....|
Expanded            TCCACATTATATGTCATGCCCTTCCATCATAAACCCAAACATTATCTCCAAAACAAGCCC 120
Original-B          ............................................................ 120
Original-FG         ............................................................ 120
Original-T          ............................................................ 120

                    130        140        150        160        170        180
                    |....|....|....|....|....|....|....|....|....|....|....|....|
Expanded            TATTCGGCCATATACCCCACCAGGCACATCCTTGTTTCAGGTACCATAAAGCCCAAGTGT 180
Original-B          ............................................................ 180
Original-FG         ............................................................ 180
Original-T          ................T...........T.......................... 180

                    190        200        210        220        230        240
                    |....|....|....|....|....|....|....|....|....|....|....|....|
Expanded            TCCTACCTACAGCCAAGCAGCAAGCGTTACCCAGAGACCAGACACTTATCAGCTATACTT 240
Original-B          ............................................................ 240
Original-FG         ............................................................ 240
Original-T          ..........G.....................A..........................  240

                    250        260        270        280        290        300
                    |....|....|....|....|....|....|....|....|....|....|....|....|
Expanded            ACAACCCAACCTAGTGAACGAGGAATATCCTAGTACACCTTTGAATTCCCCTAGTCTACA 300
Original-B          ............................................................ 300
Original-FG         ............................................................ 300
Original-T          ............................................................ 300
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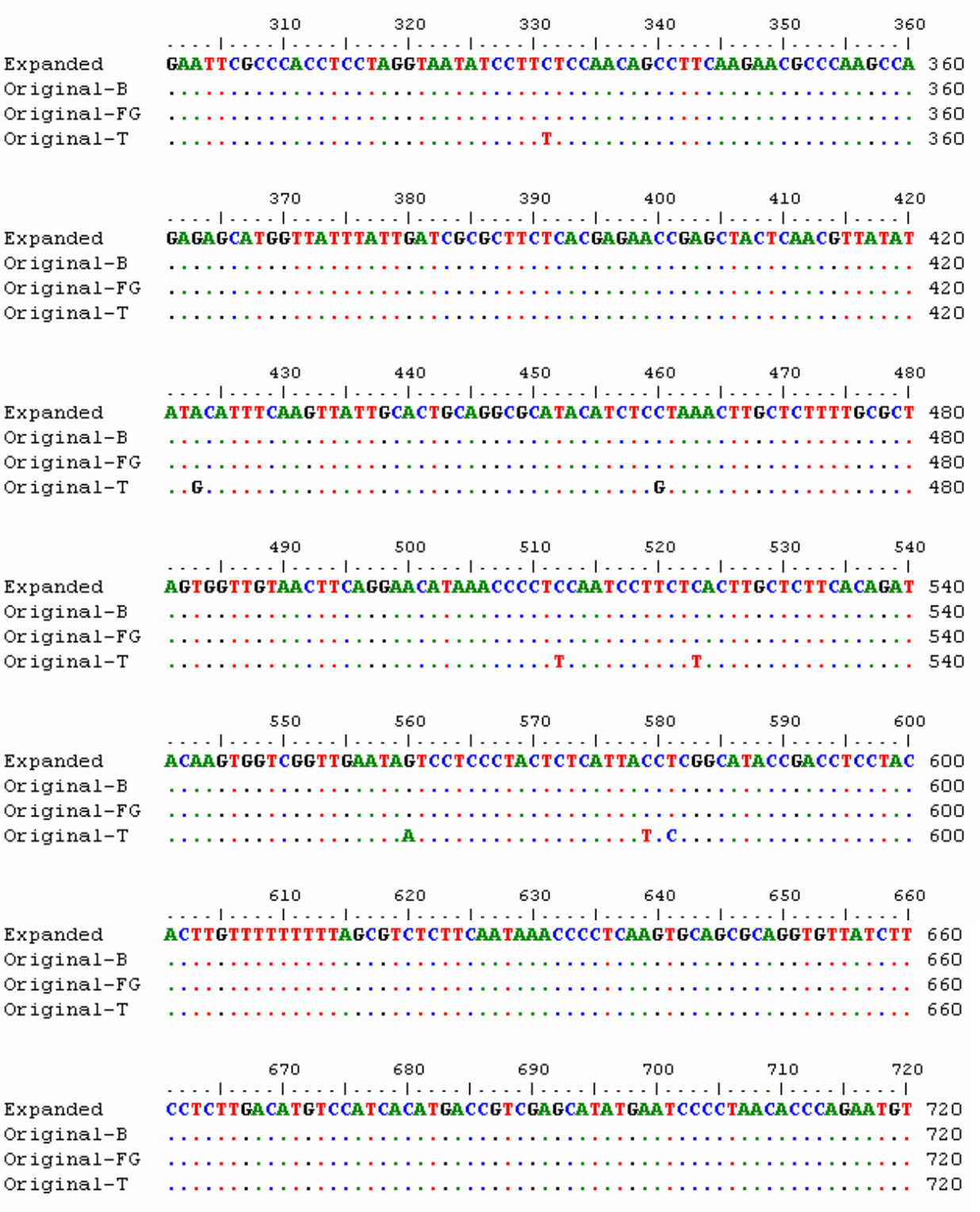

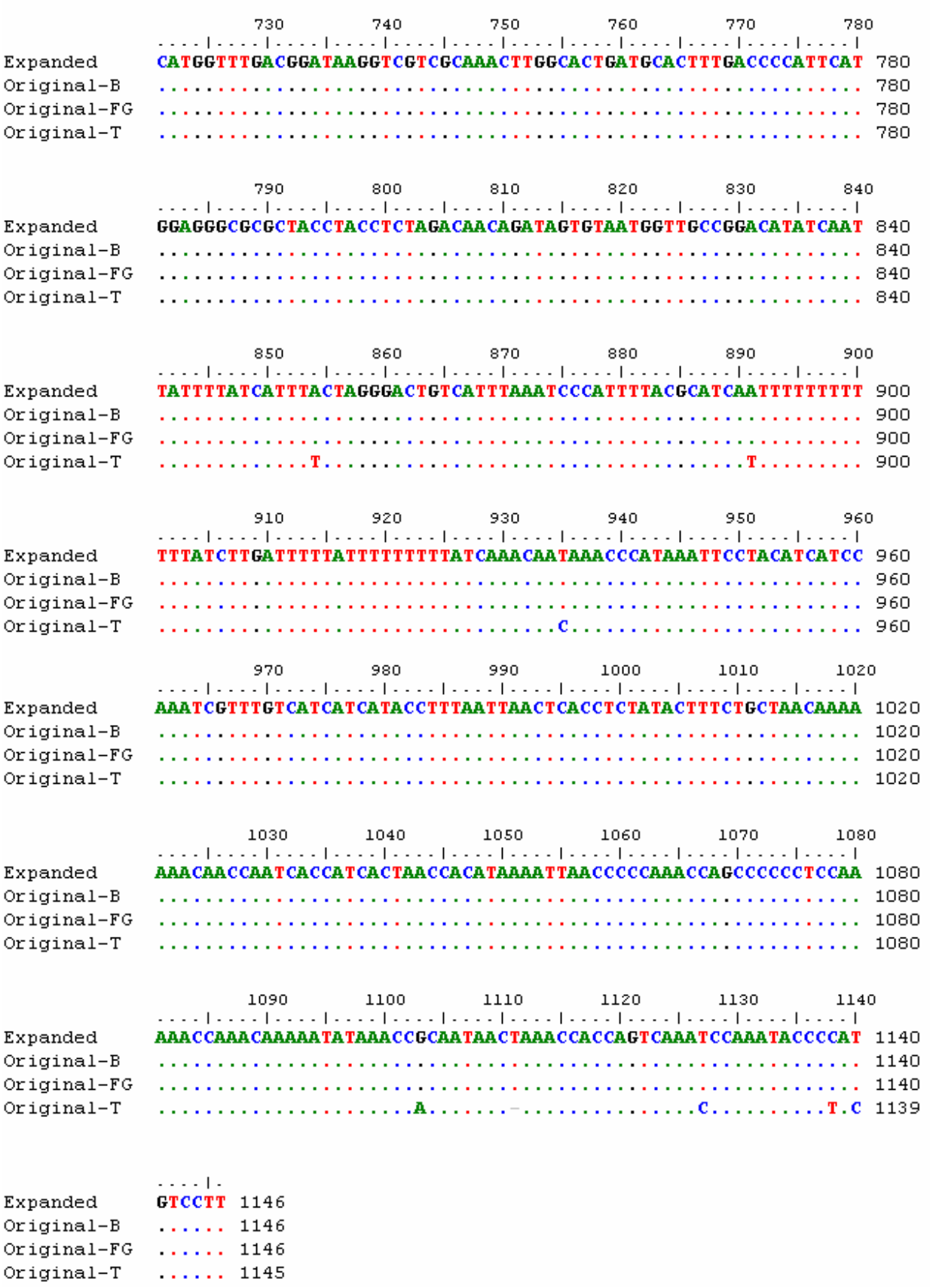

Figure 8: Shiny Cowbird *cyt b* gene nucleotide **a.** and amino acid translation **b.** sequence of expanded and original range. According Desjardins and Morais (1990) the first base corresponds to the 136th bp of the gene, and the sequence ends at the 1,023 bp of the gene.

a. *Nucleotide sequence*

```
                10        20        30        40        50        60
           ....|....|....|....|....|....|....|....|....|....|....|....|
Expanded   TATTACAGGCCTCCTACTAGCAATACATTACACAGCAGACACCAGCCTCGCCTTCTCCTC 60
Original-B ............................................................ 60
Original-FG ........................................................... 60
Original-T ............................................................ 60

                70        80        90       100       110       120
           ....|....|....|....|....|....|....|....|....|....|....|....|
Expanded   CGTAGCCCACATATGCCGAGACGTACAATTCGGCTGACTCATCCGTAACCTCCACGCAAA 120
Original-B ............................................................ 120
Original-FG ........................................................... 120
Original-T ..........................................C............... 120

               130       140       150       160       170       180
           ....|....|....|....|....|....|....|....|....|....|....|....|
Expanded   CGGAGCCTCCTTCTTCTTCATCTGCATCTACCTACACATTGGCCGAGGACTCTACTACGG 180
Original-B ............................................................ 180
Original-FG ........................................................... 180
Original-T ............................................................ 180

               190       200       210       220       230       240
           ....|....|....|....|....|....|....|....|....|....|....|....|
Expanded   CTCATACCTAAACAAAGAAACCTGAAACGTCGGAGTCATTCTCCTCCTAGCCCTAATAGC 240
Original-B ............................................................ 240
Original-FG ........................................................... 240
Original-T ..........................................C................. 240

               250       260       270       280       290       300
           ....|....|....|....|....|....|....|....|....|....|....|....|
Expanded   AACCGCCTTTGTCGGATACGTCCTACCTTGAGGACAAATATCATTCTGAGGCGCTACCGT 300
Original-B ............................................................ 300
Original-FG ........................................................... 300
Original-T ............................................................ 300

               310       320       330       340       350       360
           ....|....|....|....|....|....|....|....|....|....|....|....|
Expanded   AATCACAAACCTATTCTCAGCCATCCCATACATCGGACAAACCCTAGTAGAATGAGCCTG 360
Original-B ............................................................ 360
Original-FG ........................................................... 360
Original-T ............................................................ 360

               370       380       390       400       410       420
           ....|....|....|....|....|....|....|....|....|....|....|....|
Expanded   AGGAGGCTTCTCCGTTGATAACCCCACACTAACCCGATTCTTCGCTCTCCACTTCCTCCT 420
Original-B ............................................................ 420
Original-FG ........................................................... 420
Original-T ......G........C............................................ 420
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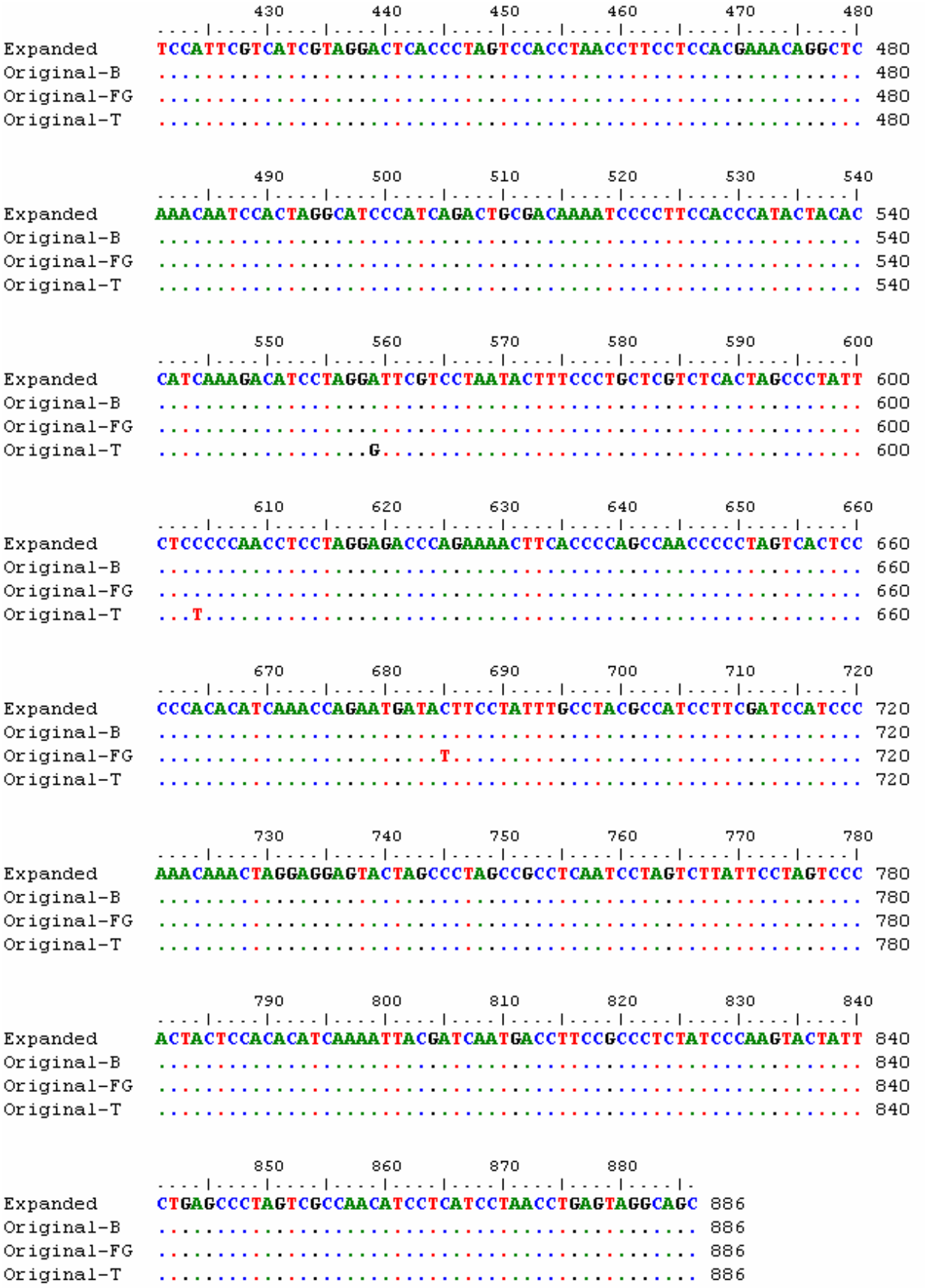

b. *Amino acid sequence.*

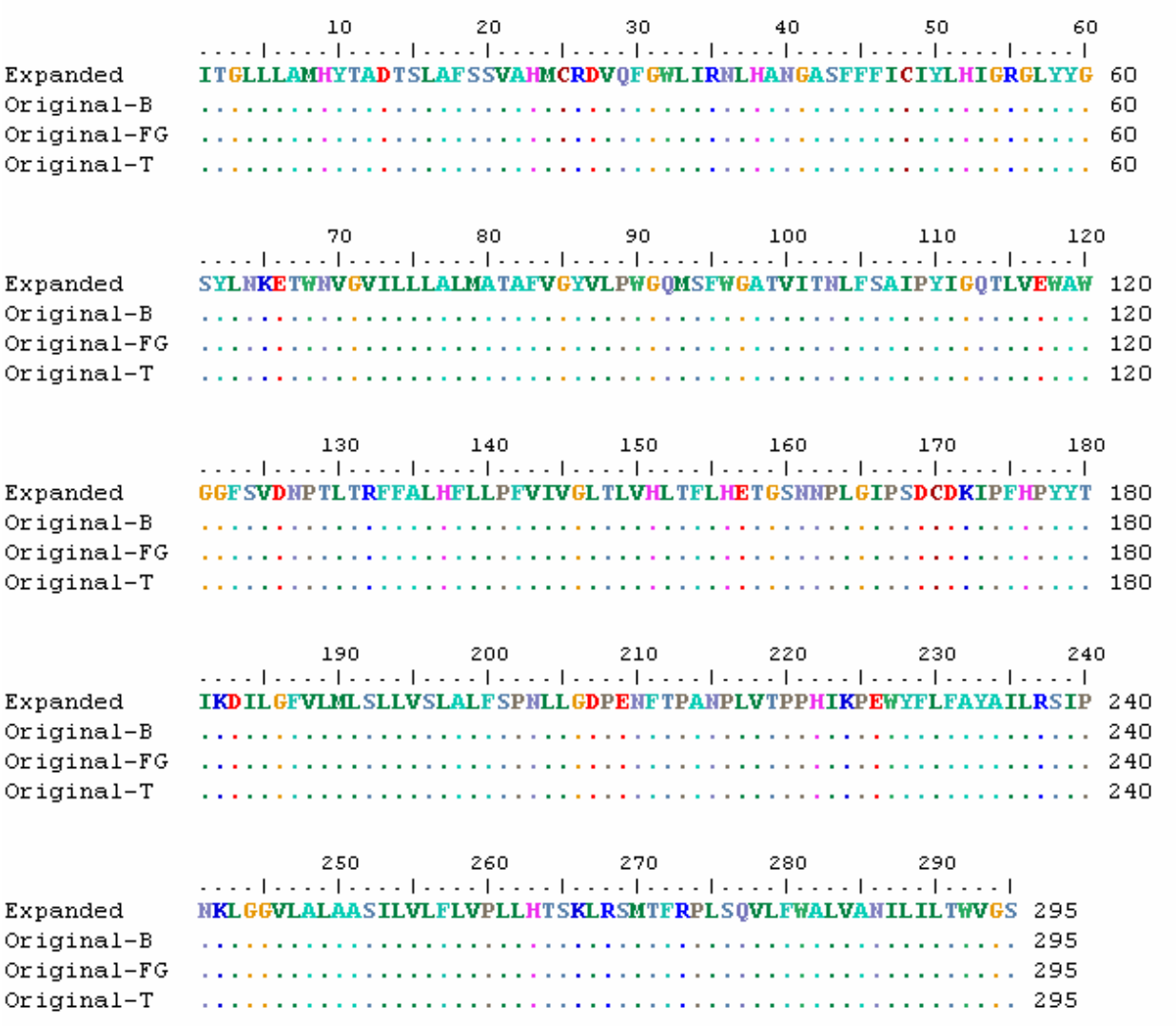

Figure 9: Shiny Cowbird *NADH2* gene nucleotide **a**. and amino acid translation **b**. sequence of expanded and original range. The first base is the 34th bp of the gene sequence. The sequence ends short by 176 bp.

a. *Nucleotide sequence.*

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                10        20        30        40        50        60
           ....|....|....|....|....|....|....|....|....|....|....|....|
Expanded   AGCCTACTTCTAGGAACAACTATCACCATCTCAAGCAACCACTGAATTATGGCCTGAGCT 60
Original-B ............................................................ 60
Original-FG ........................................................... 60
Original-T ............................................................ 60

                70        80        90       100       110       120
           ....|....|....|....|....|....|....|....|....|....|....|....|
Expanded   GGCCTTGAAATTAATACACTTGCTATCCTTCCATTAATCTCAAAATCCCACCACCCACGA 120
Original-B ............................................................ 120
Original-FG ........................................................... 120
Original-T ..................... C ..................................... 120

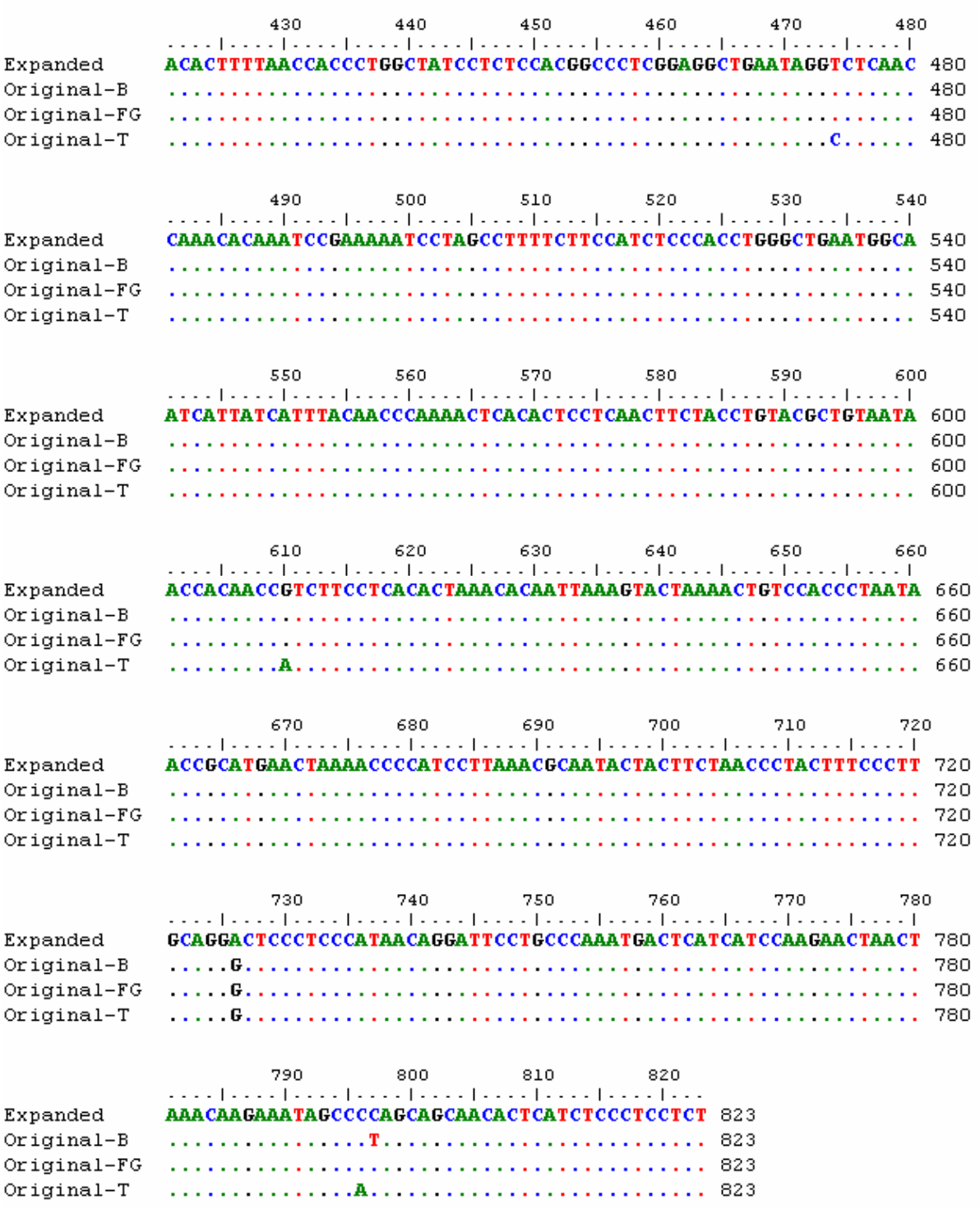
               130       140       150       160       170       180
           ....|....|....|....|....|....|....|....|....|....|....|....|
Expanded   GCCATTGAAGCTGCCACTAAATACTTCCTAACCCAAGCAGCTGCCTCCGCCCTAGTATTG 180
Original-B ............................................................ 180
Original-FG ........................................................... 180
Original-T ..........C.....................................C.. 180

               190       200       210       220       230       240
           ....|....|....|....|....|....|....|....|....|....|....|....|
Expanded   TTCTCTAGCATAACCAATGCGTGGCACACTGGACAATGAGATATCACTCAACTCACCCAT 240
Original-B ............................................................ 240
Original-FG ........................................................... 240
Original-T ..................A......................................... 240

               250       260       270       280       290       300
           ....|....|....|....|....|....|....|....|....|....|....|....|
Expanded   CCAACATCCTGCTTGATCCTCACTTCAGCAATCGCAATAAAACTAGGGTTAGTGCCATTC 300
Original-B ............................................................ 300
Original-FG ........................................................... 300
Original-T ............................................................ 300

               310       320       330       340       350       360
           ....|....|....|....|....|....|....|....|....|....|....|....|
Expanded   CACTTCTGATTCCCAGAAGTACTCCAAGGCTCTCCCCTTACCACCGGCCTACTCCTATCT 360
Original-B ............................................................ 360
Original-FG ........................................................... 360
Original-T ............................................................ 360

               370       380       390       400       410       420
           ....|....|....|....|....|....|....|....|....|....|....|....|
Expanded   ACTATTATAAAACTTCCCCCAATCACATTACTCTACATAACATCCCCATCACTAAACCCC 420
Original-B ............................................................ 420
Original-FG ........................................................... 420
Original-T ............................................................ 420
```



b. *Amino acid sequence*

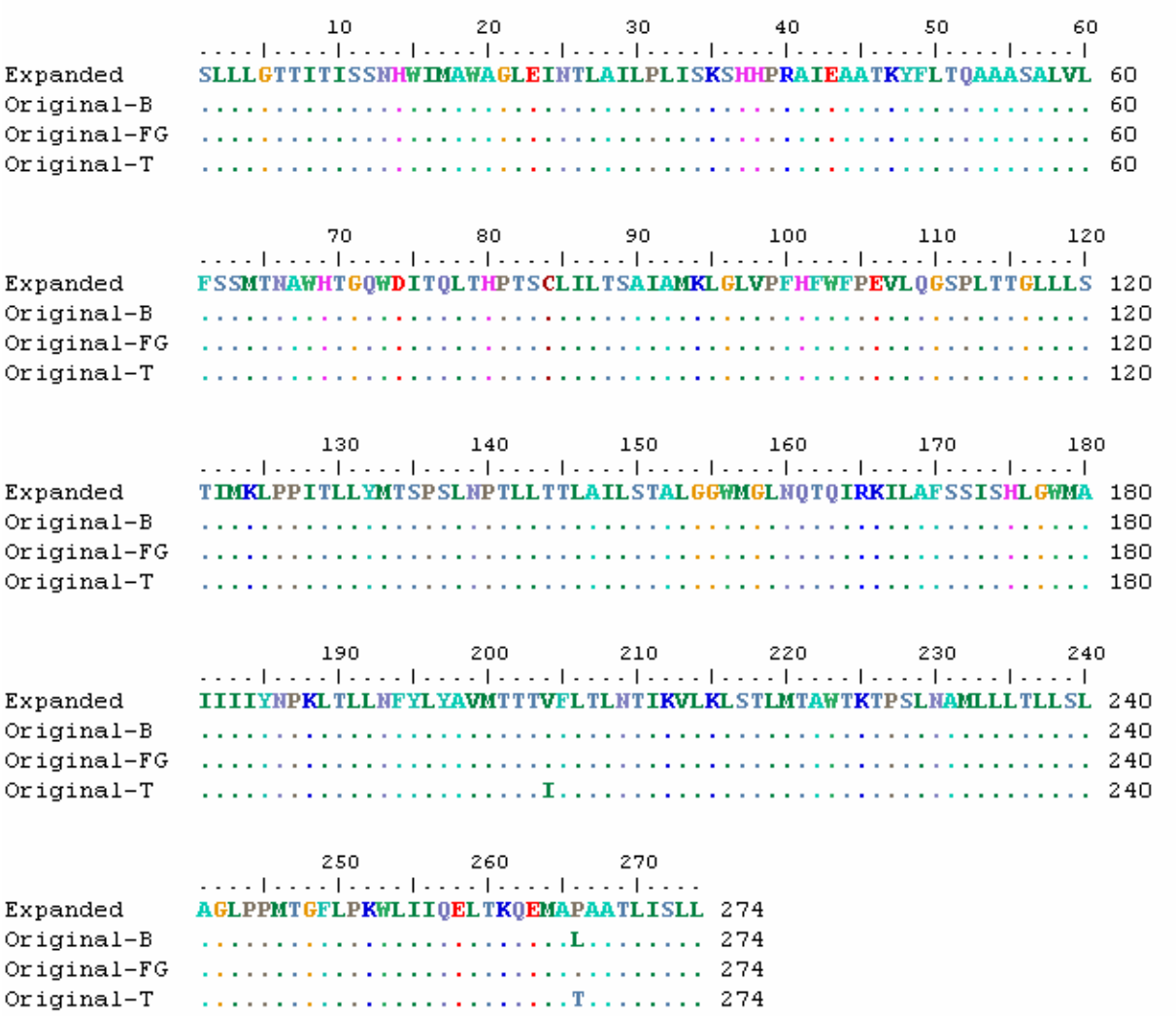


Figure 10: Complete Shiny Cowbird *NADH6* gene nucleotide **a**. and amino acid translation **b**. sequence of expanded and original range. An asterisk represents a termination codon.

a. *Nucleotide sequence*

```
                    10        20        30        40        50        60
          ....|....|....|....|....|....|....|....|....|....|....|....|
Expanded  ATGATAGAGTTTGTTCTATTTCTAGGGGTGTGTTTTGTTTTAGGGGGGTTGGCGGTTGCT 60
Original-B ............................................................ 60
Original-FG ........................................................... 60
Original-T ..........................A.........C..................... 60

                    70        80        90        100       110       120
          ....|....|....|....|....|....|....|....|....|....|....|....|
Expanded  TCTAACCCTTCCCCTTATTATGGGGTGTTAGGGCTAGTTGTAGCGGCGGTTGCGGGGTGT 120
Original-B ............................................................ 120
Original-FG ........................................................... 120
Original-T ..............................T..........................  120

                    130       140       150       160       170       180
          ....|....|....|....|....|....|....|....|....|....|....|....|
Expanded  GGTTGATTGGTGAGTTTGGGGGTTTCTTTCGTTTCTTTGGTGCTTGTCATGGTTTACTTG 180
Original-B ............................................................ 180
Original-FG ........................................................... 180
Original-T ........A.....................................................T... 180

                    190       200       210       220       230       240
          ....|....|....|....|....|....|....|....|....|....|....|....|
Expanded  GGGGGGATGCTGGTGGTGTTTGTTTATTCGGTGTCGTTAGCGGCAGATCCTTATCCGGAG 240
Original-B .........T.................................................. 240
Original-FG .........T.................................................. 240
Original-T ...........................................G.............. 240

                    250       260       270       280       290       300
          ....|....|....|....|....|....|....|....|....|....|....|....|
Expanded  TCTTGAGGTAGTTGGGGTGTTGTTGGTTATGGTTTTGGGATGGGGTTGGTTGTAGTGGTG 300
Original-B ............................................................ 300
Original-FG ........................................................... 300
Original-T G....G...................................................... 300

                    310       320       330       340       350       360
          ....|....|....|....|....|....|....|....|....|....|....|....|
Expanded  GGACTTGTTGTAGGGGGAGTGTCGGGGTTGTTGGCGGAGGATGGGACGGTGAATAATGGG 360
Original-B ....................T.................................... 360
Original-FG ....................T.................................... 360
Original-T ..G.................T.........................A.......... 360

                    370       380       390       400       410       420
          ....|....|....|....|....|....|....|....|....|....|....|....|
Expanded  GGTTTGTTGTCAGTTCGGTTGGATTTTAGTGGTGTGGCTGTGTTGTATTCGGATGGGGTT 420
Original-B ............................................................ 420
Original-FG ........................................................... 420
Original-T ............................................................ 420
```

```
                  430        440        450        460        470        480
                  ....|....|....|....|....|....|....|....|....|....|....|....|
Expanded          GGGTTGCTTTTGATTGGGGGGTGAGGGTTATTGTTAACTTTATTTGTGGTATTGGAGCTT 480
Original-B        ............................................................ 480
Original-FG       ............................................................ 480
Original-T        ...........................................................A... 480

                  490        500        510
                  ....|....|....|....|....|....|....|....
Expanded          GTGCGGGGGTTATCTCGGGGGGCGATTCGGGCTGTTTAG 519
Original-B        ........................................ 519
Original-FG       ........................................ 519
Original-T        ........................................ 519
```

b. *Amino acid sequence*

```
                  10         20         30         40         50         60
                  ....|....|....|....|....|....|....|....|....|....|....|....|
Expanded          MMEFVLFLGVCFVLGGLAVASNPSPYYGVLGLVVAAVAGCGWLVSLGVSFVSLVLVMVYL 60
Original-B        ............................................................ 60
Original-FG       ............................................................ 60
Original-T        ............................................................ 60

                  70         80         90         100        110        120
                  ....|....|....|....|....|....|....|....|....|....|....|....|
Expanded          GGMLVVFVYSVSLAADPYPESWGSWGVVGYGFGMGLVVVVGLVVGGVSGLLAEDGTVNNG 120
Original-B        ...........................................L................ 120
Original-FG       ...........................................L................ 120
Original-T        ..................A........................L................ 120

                  130        140        150        160        170
                  ....|....|....|....|....|....|....|....|....|...
Expanded          GLLSVRLDFSGVAVLYSDGVGLLLIGGWGLLLTLFVVLELVRGLSRGAIRAV* 173
Original-B        ........................................................* 173
Original-FG       ........................................................* 173
Original-T        ........................................................* 173
```

Figure 11: Complete Shiny Cowbird tRNA^{Glu} gene nucleotide sequence.

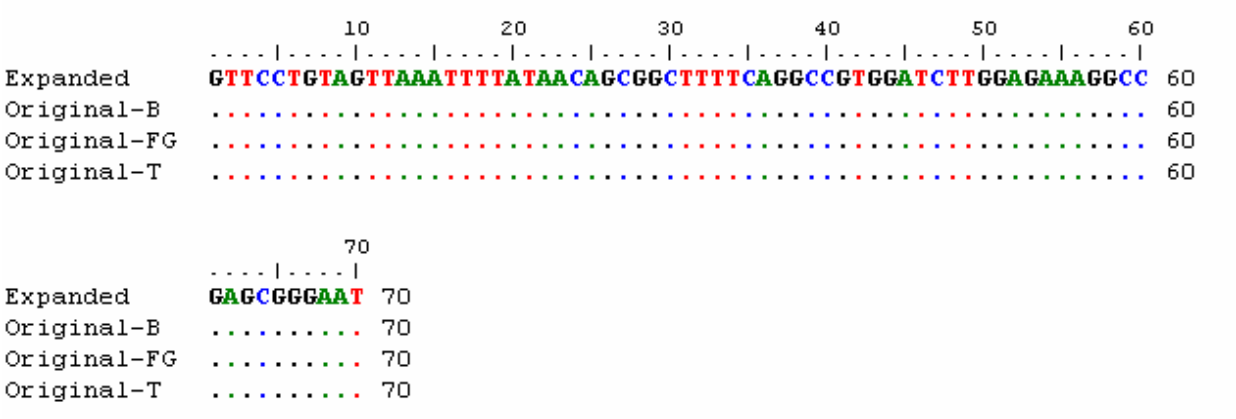
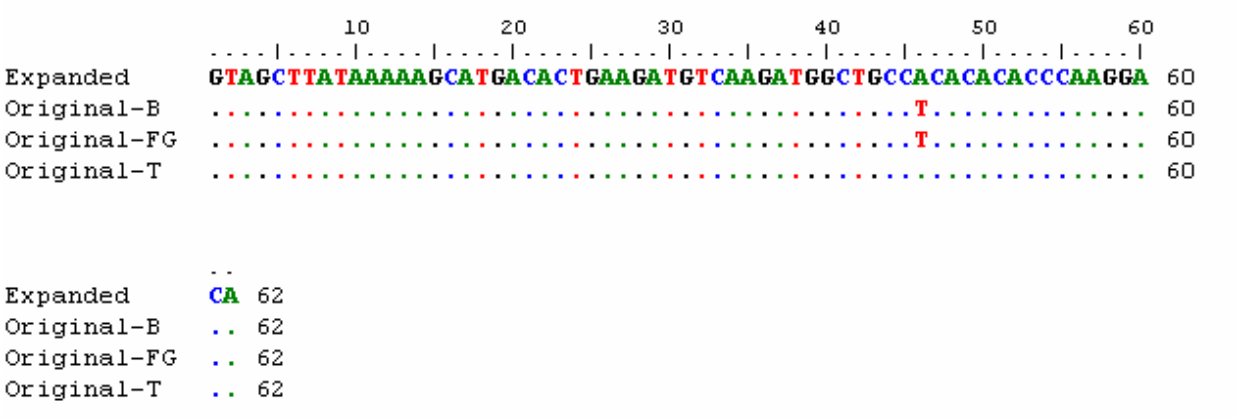


Figure 12: Complete Shiny Cowbird tRNA^{Phe} gene nucleotide sequence.



A. Analysis of sequences

The sequences of five genes and the control region generated in this investigation completed at least 78% of the genes, and 96% for the control region (Table 4). Complete sequences were achieved for *NADH6*, $tRNA^{Glu}$, and $tRNA^{Phe}$ genes.

Table 4: Comparison of the size of the genes and region to the sequenced portions.

Gene or region	Gene or region size	Sequenced portion of gene or region	
	bp	Bp	%
Control region	1,198	1,146	96
cyt b	1,140	886	78
NADH2	1,038	823	79
NADH6	519	519	100
$tRNA^{Glu}$	71	71	100
$tRNA^{Phe}$	68	68	100

The nucleotide sequence composition is presented for each gene and region in Table 5. The sequence composition of the sense strand of three out of the five genes and the light strand of the control region shows a high pyrimidine/purine ratio. The higher purine/pyrimidine ratio of the *NADH6* gene is explained by its coding in the opposite direction. However, it is noteworthy that the pyrimidine /purine ratio of $tRNA^{Phe}$ run contrary to the expectation based on the direction of their transcription. For all the genes but $tRNA^{Phe}$ the G-C difference is larger than the A-T difference. The *NADH6* gene has a considerably different nucleotide composition to all other genes and regions in that it has a higher quantity of guanine and thiamine in both original and expanded range.

Table 5: Percent nucleotide composition of the sense strand of the sequenced genes and the light strand of the control region.

Gene or region	Expanded				Original[1]				Py/Pu[2]
	A	G	C	T	A	G	C	T	
Control region	29.58	13.51	28.88	28.03	29.99	13.59	27.95	28.47	57/43
cyt b	28.17	13.08	35.31	23.44	27.20	13.45	35.89	23.46	58/42
NADH2	30.57	10.29	34.97	24.18	30.65	10.60	34.66	24.09	58/41
NADH6	12.83	40.00	10.00	37.17	12.58	39.72	9.48	38.21	48/52
$tRNA^{Glu}$	24.29	30.00	17.14	28.57	24.29	30.00	17.14	28.57	46/54
$tRNA^{Phe}$	37.10	20.97	24.19	17.74	37.63	20.97	24.19	17.30	42/58

[1]Average between samples of Brazil, French Guiana, and Trinidad.
[2]Py/Pu=Pyrimidine/Purine

The transitions and transversions observed in the sequenced genes and regions are presented in Table 6. Polymorphic sites ranged from 2.50% in the *NADH6* genes to 0% in $tRNA^{Glu}$. The highest number of changes in the sequences was in the control region with 21, four of which were transversions. Up to eleven synonymous (silent mutations) were detected in the amino acid sequence. The highest was in *NADH6* genes, followed with seven in *cyt b* and six in *NADH2* genes. In addition, three and two nonsynonymous (replacement) changes were identified in the *NADH2* and *NADH6* gene amino acid sequences, respectively.

Table 6: Transitions (Ti) and transversions (Tv) in synonymous and nonsynonymous sites of the sequenced genes and regions.

Gene or Region	Nucleotide				Synonymous			Nonsynonymous		
	P[1]	Ti	Tv	T[2]	Ti	Tv	T[2]	Ti	Tv	T[2]
Control region	1.83	17	4	**21**	---	---	---	---	---	---
cyt b	0.79	6	1	**7**	6	1	7	0	0	0
NADH2	1.09	8	1	**9**	6	0	6	2	1	3
NADH6	2.50	11	2	**13**	11	0	11	0	2	2
$tRNA^{Glu}$	0	0	0	**0**	---	---	---	---	---	---
$tRNA^{Phe}$	1.61	1	0	**1**	---	---	---	---	---	---
$\overline{\chi}$	1.30	43	8	51	23	1	24	2	3	5

[1]Percentage of polymorphic sites (segregating sites/ total sequenced bps); [2]Total

Amino acid variability within the codon position was also analyzed (Table 7). Third positions of the codons reflected higher variability than the first and second positions. There were twenty-eight C/T or T/C and fifteen G/A or A/G transitions observed in the total sequenced genes and regions from the sense strand. However, fourteen C/T and twelve G/A were found in the protein-coding sequences. It is noticeable that most transitions occurred in the third codon position. In addition, two transversions occurred on the first codon position, and only one was observed at the third position in the *cyt b* gene.

Table 7: Codon position variability of the sequenced genes and region.

Gene	Codon position	Ti		Tv[2]	Total
		C/T[1]	G/A[1]		
cyt b	1	0	0	0	0
	2	0	0	0	0
	3	5	1	C/G	7
NADH2	1	1	1	C/A	3
	2	0	1	0	1
	3	3	2	0	5
NADH6	1	3	0	T/G	4
	2	1	0	0	1
	3	1	7	0	8

[1]Or vice versa; [2]Expanded/Original

IV. Genetic diversity

Genetic diversity estimates obtained for the sequenced genes and regions of interest are presented in Table 8. Estimates included specimens from the expanded and original range, except Trinidad. This sample reflects a much higher nucleotide sequence divergence that suggests it does not correspond to the other *M. b. minimus*. Hence, it will not be counted for in the estimates because it may introduce high estimate error.

Only limited estimates were available due to the low quantity of haplotypes found within the sampled specimens. For instance, the control region and $tRNA^{Glu}$ gene had only one allele and thus were not measurable in any of the proposed estimates of genetic diversity. Likewise, the *cyt b* and *NADH6* genes, yielded two haplotypes and these did not produce measurable $\hat{H}$ and F_{ST}. $\hat{H}$, the measurement of genetic variation in a population, was only measurable in the *NADH2* gene, which presented three haplotypes. The population fixation index average estimate resulted in 0.85772. $\hat{\pi}$, a nucleotide diversity estimate, reflected an average of 0.00043. The highest estimate was for $tRNA^{Glu}$ with 0.00124, and the lowest measurable estimate was 0.00006 for *cyt b*. *K*, a standardized estimate of segregating sites adjusted to the length of sequence, resulted with an average of 0.00386. It is noticeable that the highest difference between K and $\hat{\pi}$ estimates are in the *NADH6 gene*.

Table 8: Genetic diversity estimates of the Shiny Cowbirds sampled populations.

Gene or Region	$\hat{H}$	F_{ST}	$\hat{\pi}$	K
Control region	---	---	---	---
cyt b	---	---	0.00005931	0.00027037
NADH2	0.66666667	0.711543094	0.00012588	0.00058213
NADH6	---	---	0.00029509	0.00092311
$tRNA^{Glu}$	---	---	---	---
$tRNA^{Phe}$	--	1.0	0.00123511	0.00386366
$\overline{\chi}$	0.66666667	0.85771547	0.00042885	0.00141000
total	0.66666667	0.65385171	0.00011319	0.00042161

V. Phylogenetic trees

Additive phylogenetic trees were constructed using Mega 3.1 (Kumar *et al.* 2004) to elucidate the degree of divergence between the SHCO subspecies analyzed in this investigation. The trees were constructed using the nucleotide substitution model with the Kimura 2 parameter, and the Bootstrap test with Minimum Evolution method (Kumar 1996) with 5,000 replicates.

Sequences of other *Molothrus* available in GeneBank were included to support genetic distances between the species (Table 9). Icterids species used as outgroups for the *cyt b* and *NADH2* analysis were *Dives warszewiczi* (Scrub Blackbird)*, Icterus bullockii* (Bullock's Oriole), and *Quiscalus major* (Boat-tailed Grackle). In addition, an Emberizidae sequence of *Junco hyemalis* (Dark-eyed Junco) was used for *cyt b*, *NADH2*, *NADH6* genes. Phylogenetic trees were not constructed for the control region, $tRNA^{Glu}$, and $tRNA^{Phe}$ due to limited sequences available *in silico* to compare those established in this investigation.

It is important to note that the sequences for *cyt b* and *NADH2* genes available in GeneBank are of poor quality because they presented undistinguished nucleotides (e.g. n, r, or y). Consequently, twenty-one nucleotide sites were omitted in the sequence analysis in order to reduce ambiguity in the calculation of the phylogenetic trees. Also, eleven nucleotides from the *NADH2* tree were omitted, due to the poor quality sequence AY138936 (Fig. 14).

Table 9: GeneBank accession numbers of sequences used to construct phylogenetic trees for the *cyt b* *NADH2* and *NADH6* genes.

	Species	GeneBank accession numbers		
		cyt b	***NADH2***	***NADH6***
Ingroup	*Molothrus aeneus aeneus*	AF089040	AF109957	---
	M. ater ater	AF290172 AF089041	AF447291 AF109958	---
	M. bonariensis minimus	AF089043	AF109959	----
	M. bonariensis occidentalis	---	AF407047	AF407133
	M. oryzivorus oryzivorus	AF089060	AF407046 AF109960	AF407132
	M. rufoaxillaris	AF089044	AF109961	---
Outgroup	*Dives warszewiczi*	AF089021	AF109962	---
	Icterus bullockii	AF290167	AF290130	---
	Quiscalus major	AF290171	AF109953	---
	Junco hyemalis	AF290161	AY138936	AF407130

Figure 13: Minimum evolution additive tree of the 835 bp of the *cyt b* gene sequences for *Molothrus* and outgroups (*Quiscalus*, *Dives, and Icterus).* The scale represents 1 substitution per 100 nucleotides. Significant bootstrap values are indicated above the node.

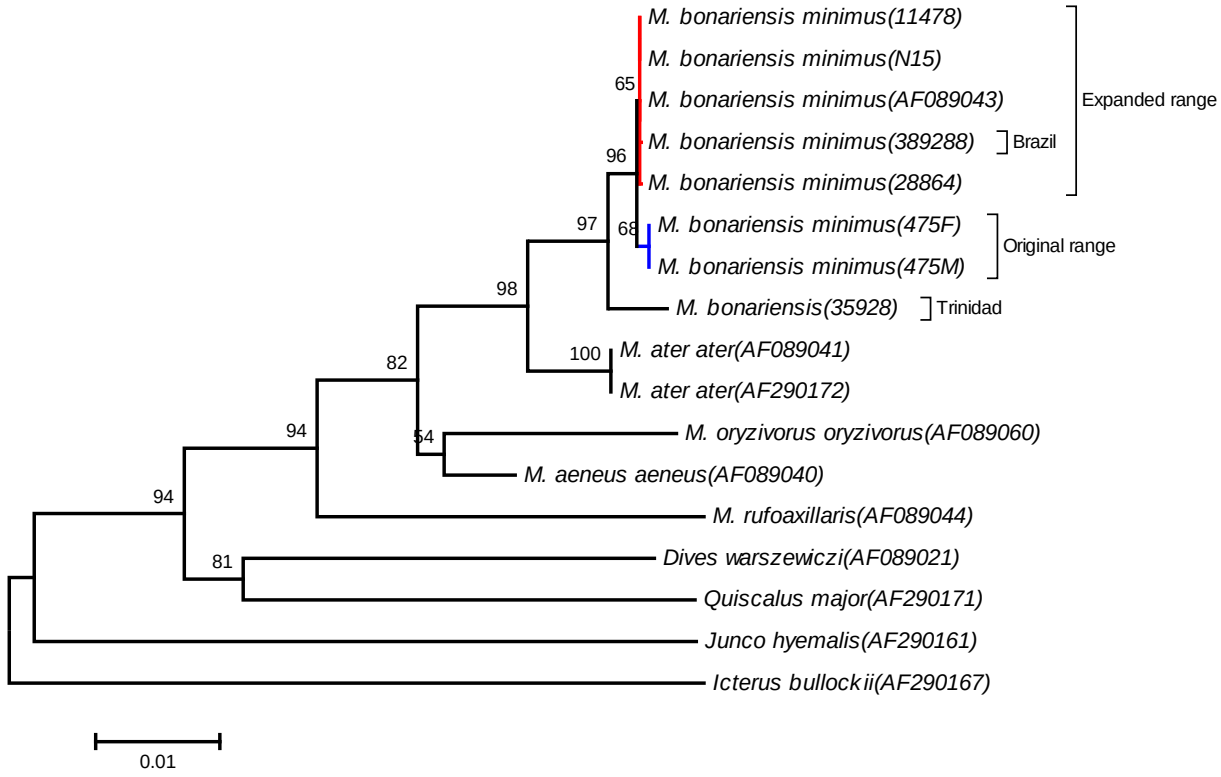


Figure 14: Minimum evolution additive tree for 819 bp of the *NADH2* gene sequences of *Molothrus* and outgroups (*Dives, Icterus, Junco, and Quiscalus).* The scale represents 2 substitutions per 100 nucleotides. Significant bootstrap values are indicated above the node.

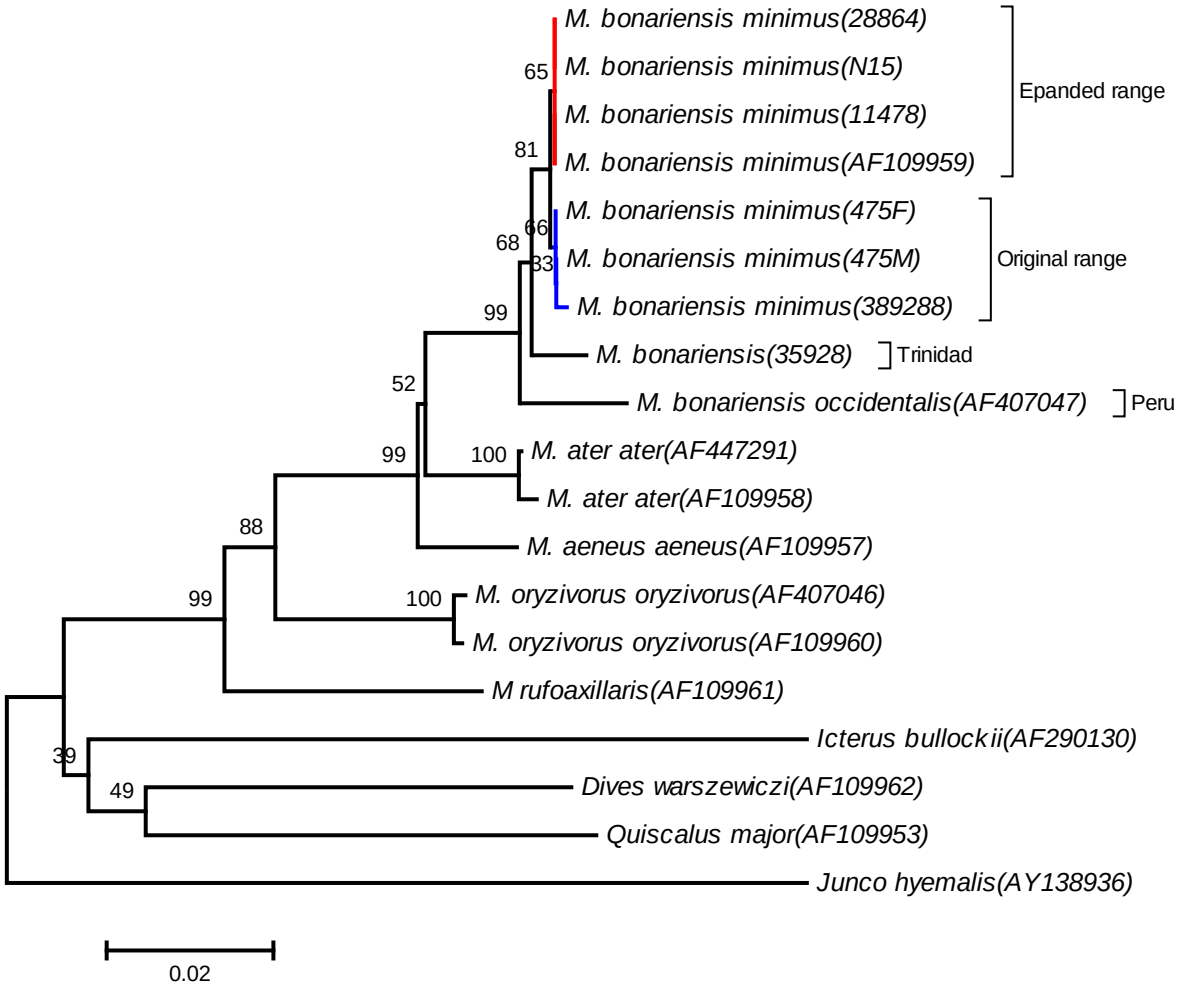


Figure 15: Minimum evolution additive tree for 477 bp of the *NADH6* gene sequences for *Molothrus* bonariensis and *M. oryzivorus,* and *Junco* as outgroup. The scale represents 2 substitutions per 100 nucleotides. Significant values are indicated above the node.

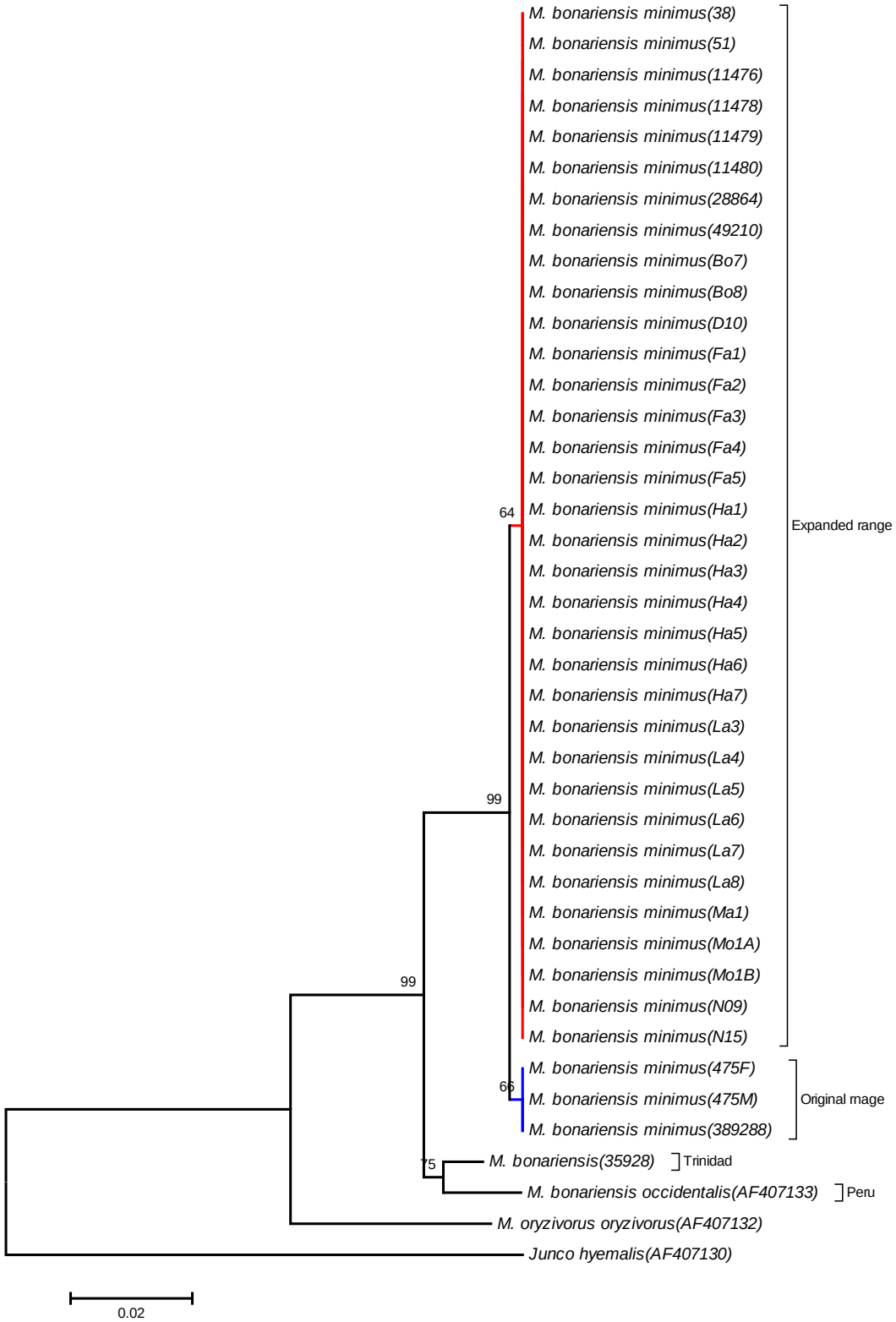


Figure 16: Minimum Evolution additive tree of *cyt b* and *NADH2* genes (1,498 bp) for *Molothrus* species and *Dives, Icterus, Junco,* and *Quiscalus* as outgroups. The scale represents 1 substitution per 100 nucleotides.

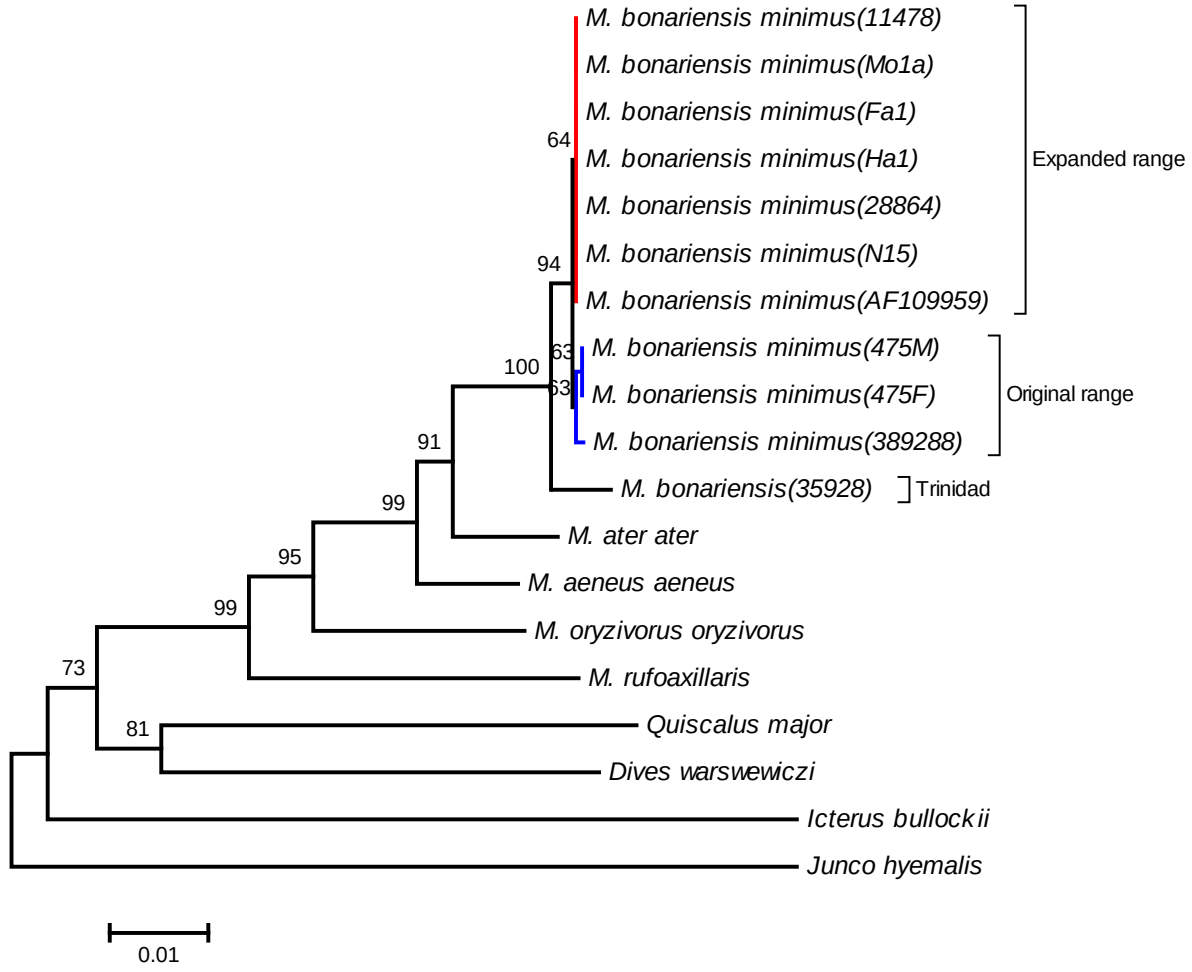


Figure 17: Minimum Evolution additive tree of *NADH6* and *cyt b* sequence genes (1,146 bp) for *Molothrus bonariensis* species and *Junco hyemalis* as outgroup. The scale represents 5 substitutions per 100 nucleotides.

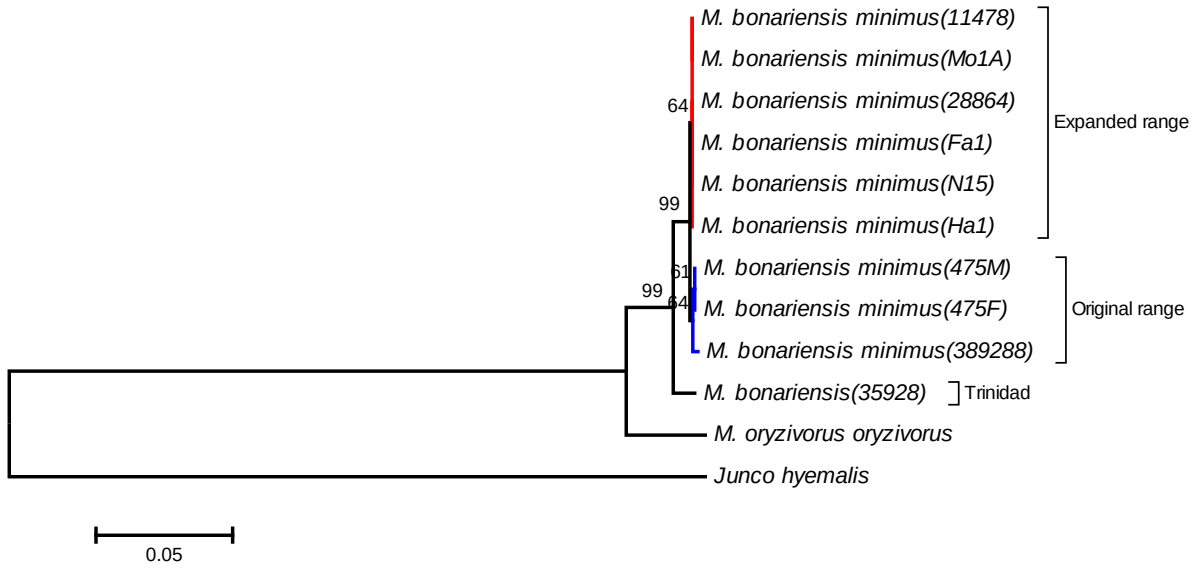


Figure 18: Minimum Evolution additive tree of *NADH2* and *NADH6* sequence genes (1,301 bp) for *Molothrus bonariensis* species and *Junco hyemalis* as outgroup. The scale represents 2 substitutions per 100 nucleotides.

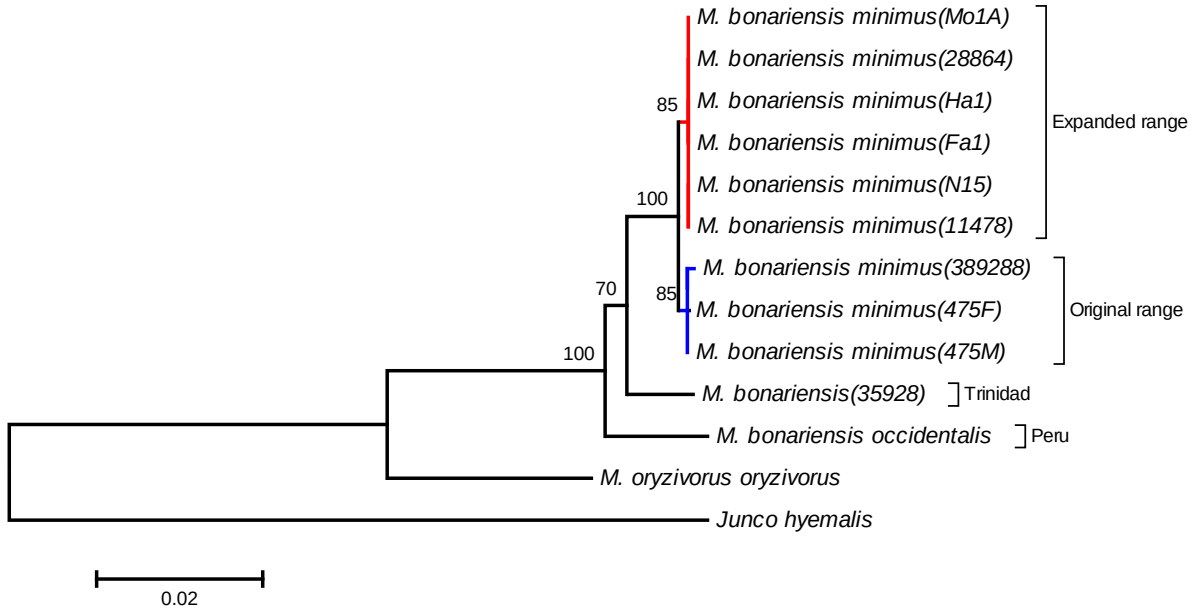
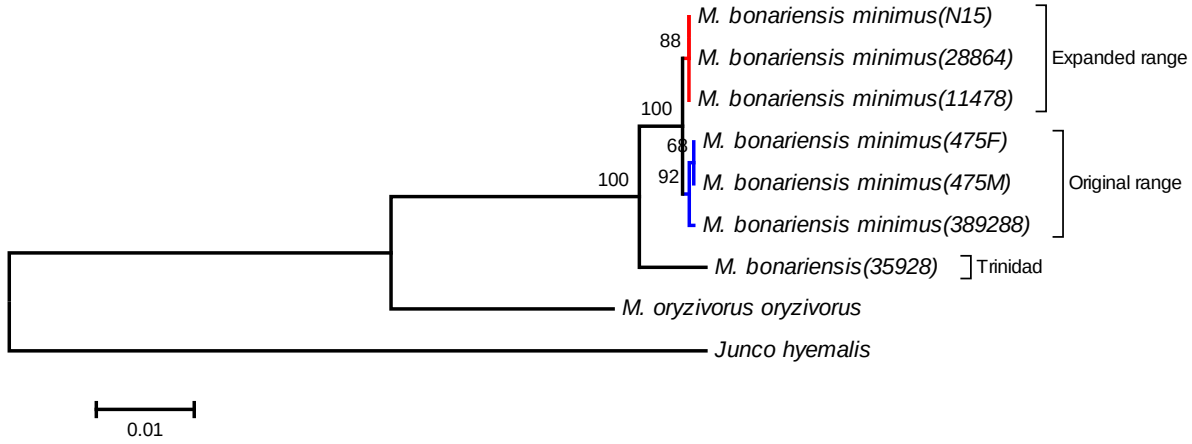
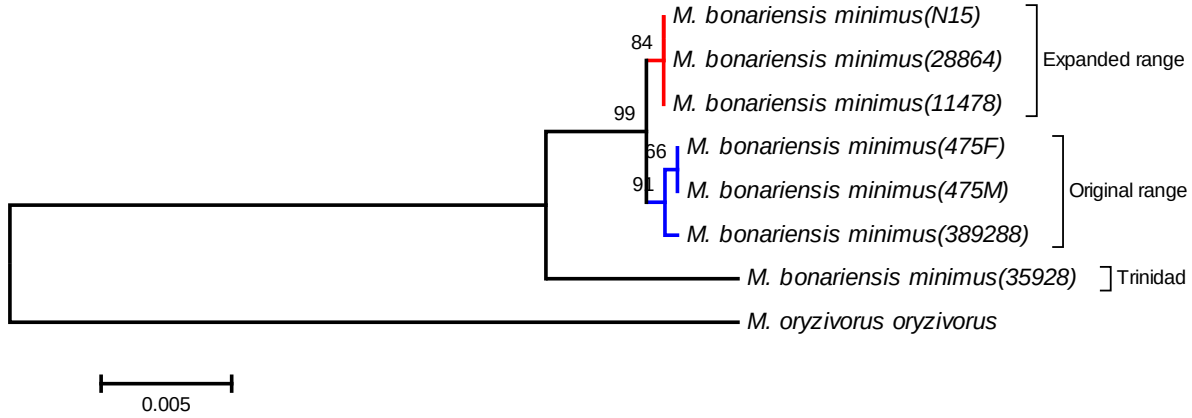


Figure 19: a. Minimum Evolution additive tree of *NADH2*, *NADH6* and *cyt b* sequence genes (2,167 bp) for *Molothrus bonariensis* species and *Junco hyemalis* as outgroup. The scale represents 1 substitution per 100 nucleotides. **b**. Expansion of additive tree without *Junco hyemalis* sequence (2,169 bp). The scale represents 5 substitutions per 1,000 nucleotides. **c**. Expansion of the tree without *M. oryzivorus* and *Junco hyemalis* sequences (2,173 bp). The scale represents 1 substitutions per 1,000 nucleotides.

a.



b.



c.

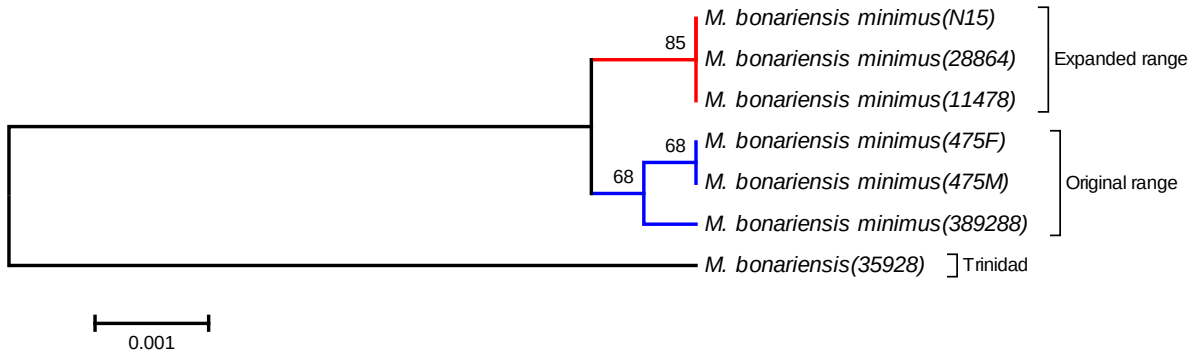


Table 10: Pairwise distance matrix of the *NADH2*, *NADH6* and *cyt b* genes.

	M. b. minimus(11478)	***M. b. minimus(28864)***	***M. b. minimus(Ha1)***	***M. b. minimus(MO1a)***	***M. b. minimus (N15)***	***M. b. minimus(Fa1)***	***M. b. minimus(475F)***	***M. b. minimus(475M)***	***M. b. minimus(389288)***	***M. bonariensis (35928)***	***M. o. oryzivorus***	***Junco hyemalis***
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1												
2	0.0000											
3	0.0000	0.0000										
4	0.0000	0.0000	0.0000									
5	0.0000	0.0000	0.0000	0.0000								
6	0.0000	0.0000	0.0000	0.0000	0.0000							
7	0.0020	0.0020	0.0020	0.0020	0.0020	0.0020						
8	0.0020	0.0020	0.0020	0.0020	0.0020	0.0020	0.0000					
9	0.0025	0.0025	0.0025	0.0025	0.0025	0.0025	0.0015	0.0015				
10	0.0051	0.0051	0.0051	0.0051	0.0051	0.0051	0.0041	0.0041	0.0046			
11	0.0531	0.0531	0.0531	0.0531	0.0531	0.0531	0.0042	0.0042	0.0548	0.0564		
12	0.1443	0.1443	0.1443	0.1443	0.1443	0.1443	0.1449	0.1449	0.1456	0.1449	0.1375	

DISCUSSION

I. Sequence data

Eleven populations were sampled for this investigation, including 34 individuals from the expanded range and 4 from the original range. The expanded range consisted of thirty-two specimens from Puerto Rico, one from Louisiana, and another from Florida, representing 89% of the samples. Meanwhile, the original range consisted of one specimen each from Trinidad and Brazil, and two from French Guiana, representing 11% of the samples. A total of 3,513 bp of mtDNA was sequenced for each SHCO sampled specimen. The sizes of the genes obtained in this investigation are comparable to those established for the chicken by Desjardins and Morais (1990), with the exception of the control region that is 29 bp shorter than in the chicken. If the mtDNA genome size of the SHCO is assumed to be similar to that of the chicken then, the sequenced portions represent 22%.

According to Desjardins and Morais (1990), the chicken mtDNA molecular organization presents the *NADH6* gene located between the $tRNA^{Pro}$ and $tRNA^{Glu}$ genes and the control region between the $tRNA^{Glu}$ and $tRNA^{Phe}$ genes. By contrast, in mammals (*e.g.* human, cow, mouse, and rat) and in the African clawed frog (*Xenopus laevis*) the *NADH6* gene is located between the $tRNA^{Glu}$ and NADH5 genes, and the $tRNA^{Pro}$ is adjacent to the control region. Interestingly, the rearrangement of the *NADH6* gene in birds joins the transcription of the $tRNA^{Glu}$ and *NADH6* genes to that of the $tRNA^{Pro}$ in a single transcription unit. The molecular organization of the sequenced mitochondrial genes of the SHCO agrees with that established by Desjardins and Morais (1990).

A. Genes and control region

The sequences obtained from the 34 sampled individuals in the expanded area were identical. This is a remarkable finding because it suggests that it is the only matrilineage represented in the Greater Antilles since at least 1987. The prevalence of only one allele in the expanded range denotes that range expansion occurred recently, because no mutations have accumulated in any of the sampled populations since then. Fleischer and other collaborators (1998) estimated a divergence rate in bird mtDNA of 2% per million years. Thus, no mutations occurring in the past century from the founding haplotype can reasonably be expected.

On the contrary, the four samples obtained from the original range presented three distinct sequences. Even though the sequences from both specimens of French Guiana presented the same allele, a higher genetic diversity is present at the original range in the sequenced mitochondrial genes and regions. Yet, it was not expected that the sequences from French Guiana and Brazil were more similar to that of the expanded range than Trinidad. The specimens from French Guiana and Brazil have control region sequences identical to those of the expanded range.

Five differences (0.142%) were observed on all 3,513 sequenced bp nucleotides between the expanded and French Guiana specimens. The same number of differences applied to the specimens in the expanded range when compared to the specimen of Brazil. Yet, the differences within the continental specimens were not the same. Between the continental specimens, only two differences were found in common, thus, reflecting a divergence of 0.056%. Initial expectation dictated that the continental samples from French Guiana and Brazil were to resemble that of Trinidad. However, a difference of 48 (1.366%) nucleotides was observed between the continental specimens and Trinidad. In addition,

when Trinidad is contrasted to the expanded range sequences it differed by 47 (1.337%) nucleotides. Of the noted differences, five entail nonsynonymous changes. Some similarities exist within the nonsynonymous changes. For instance, in the *NADH6* protein sequence at position 108, all three original range sequences reflected the alteration of leucine into serine in the expanded range, suggesting a nonsynonymous mutation in the lineage leading to the expanded range. In addition, at position 266, in the *NADH2* protein sequence, Brazil reflected leucine, and Trinidad threonine, meanwhile French Guiana and all samples from the expanded range indicated proline. These changes may imply considerable modifications in the protein structure because of the alteration in the non-conservative nature of these amino acid changes.

I. Control region

The non-coding control region is a sequence rich in genetic markers, yet in this investigation, it has proven not to be particularly highly informative, probably due to the limited time that has occurred since the subspecies settled in the novel expanded range. In humans, the control region contains two hypervariable sections. This characteristic has allowed the study of migration patterns by the establishment of distinct haplotypes in a relatively short period. In birds, however, the control region has not served this function.

The control region in birds is quite variable; the shortest documented so far has been that of the Dunlin (*Calidris alpina*) with 1,072 bp and the largest that of the Greenfinch (*Carduelis chloris*) with 1,240 bp (Baker and Marshall 1997). The SHCO control region bp length is of 1,198. According to *M. bonariensis occidentalis* sequences in GenBank (AF407133), the first 57 bp of the control region were not amplified in this investigation.

The nucleotide frequency is comparable to the average obtained by other investigations (Baker and Marshall 1997). Sequences provided by this investigation reflect a low percentage (14%) of guanine in the light strand, while the composition of the remaining nucleotides averages 29%. Investigators have divided the control region into three domains, in which it has been found that the third is most variable (*op. cit.*). However, in the SHCO, the variable sites along the control region sequences were evenly distributed with seven point mutations on each domain.

No differences were found between the sequences of the expanded range and those of French Guiana and Brazil. Twenty-one differences were observed when the sequences of the expanded range specimens were compared to that of the original range from Trinidad. Four of these were transversions and the seventeen were transitions. In addition, one base pair deletion was observed in the control region sequence of Trinidad when compared to the expanded Brazil and French Guiana sequences at position 1,111.

II. Cytochrome b

Pioneering work by Kocher *et al.* (1989) provided universal primers for a wide range of vertebrate animals. *Cyt b* is the most investigated mitochondrial gene in birds (Moore and DeFilippis 1997, Sorenson *et al.* 1999). Even though, the *cyt b* gene has been suggested to not necessarily be the most informative because most of its variation is synonymous (Desjardins and Morais 1990, Moore and DeFilippis 1997). Consequently, a reliable source of information might be found in other protein-coding genes that have higher nonsynonymous substitution rates.

This investigation reveals that the *NADH2* and *NADH6* genes have higher nonsynonymous substitution rates than *cyt b*. Therefore, the conserved *cyt b* gene

sequences resulted in the lowest polymorphic rate among the investigated genes and regions with 0.79%. This evidence supports the limited usefulness of *cyt b* gene sequences at the subspecies level.

The average nucleotide base composition, as in the control region, reflected 14% for guanine in the light strand, and 29% for each of the remaining nucleotides. Three haplotypes were identifiable. The amino acid sequence provided by this investigation did not present any nonsynonymous change.

III. NADH2

After *cyt b*, *NADH2* is conceivably the most readily available *Molothrus* gene sequence at GeneBank. The sequence of AF109959 from a *M. bonariensis minimus* is identical to that obtained for the expanded range sequences. This is not surprising because this specimen was collected in Cabo Rojo, Puerto Rico in 1987.

It is important to note that in this gene the nucleotide base composition reflects the lowest concentration (10%) of guanine in the light strand among all the sequenced genes and regions, and an average of 30% for each of the remaining nucleotides.

Nine polymorphic sites and four haplotypes were identifiable in the *NADH2* gene. An interesting finding is the identity in the nucleotide sequence in the original range at position 726; it differs in the expanded range. Change resulted in the substitution of guanine in the original sequences for adenine in the expanded sequences, but it does not alter the amino acid sequence. Eight other changes are observed within the nucleotide sequences; of these, three generate modifications in the amino acid sequences. At position 204, of the amino acid sequence, isoleucine converts into valine in the expanded and French Guiana sequences. This conversion

results in the loss of most of the side chain. In position 267, Trinidad presented threonine and it is modified into proline in the expanded range sequences as in French Guiana, but in Brazil, it is converted into leucine. It represents a change in neutral hydrophilic amino acid into a neutral hydrophobic amino acid sequence. The amino acid sequences provided by this investigation present four haplotypes, one corresponding to each country within the original range and another for the 34 specimens, of the expanded range. These findings reflect that the *NADH2* gene has higher polymorphism than the conserved *cyt b* gene.

IV. NADH6

NADH6 has been poorly investigated within the *Molothrus* genus; even at the Icteridae level, only two sequences are available at GeneBank. The *NADH6* and ATPase 8 genes were found to be the most divergent sequences in the chicken mtDNA as compared to mammals (Desjardins and Morais 1990). Hence, in closely related taxa these may serve as more reliable sequences of phylogenetic separation.

This investigation provided the complete sequence of the *NADH6* gene; it resulted with the highest frequency of polymorphic sites with 2.50%, and three haplotypes. As in *NADH2*, a difference between the original range and the expanded sequences was found. In which at position 323, original range specimens revealed thymine and the expanded range sequences indicated cytosine. This transition is nonsynonymous and causes the change, at position 108 in the amino acid sequence, of serine to leucine. This modification implies the loss of two methyl groups in the side chain of the amino acid and the addition of a hydroxyl group, thus, converting a neutral hydrophobic amino acid into a neutral β-turning hydrophilic amino acid. Perhaps, this represents a continental amino acid sequence

ancestral character. Another nonsynonymous change present in the Trinidad sequence was observed at position 81. The modification consisted on the conversion of alanine into serine. This modification entails another replacement of a neutral hydrophobic amino acid with a neutral hydrophilic amino acid.

When compared to the partial sequence AF407133 of *M. bonariensis occidentalis*, of the 474 bp in common, 19 nucleotide changes were observed. Of the 158 amino acids aligned, three nonsynonymous changes were noted. The amino acid sequences provided by this investigation present three haplotypes, one corresponding to Trinidad, a second to the continental specimens and another to the 34 specimens of the expanded range. These findings reflect that the *NADH6* gene has higher polymorphism than the conserved *NADH2* and *cyt b* genes.

V. tRNA^{Glu}

The complete sequence of tRNA^{Glu} is provided in this investigation, and no modifications were observed within the original or expanded sequences. However, when compared to sequence AF407133 of *M. bonariensis occidentalis*, the alignment reflected two changes of purines at positions 26 and 55. The available amino acid sequence presented one haplotype for all the sequenced specimens.

VI. tRNA^{Phe}

The complete sequence of tRNA^{Phe} is provided in this investigation. A transversion was observed within the continental sequences. At position 46, a thymine was observed, in the continental specimens, meanwhile the sequence of Trinidad and those of the expanded range presented adenine. In addition, when compared to the partial sequence AF407133 of *M. bonariensis occidentalis*, the alignment of 35 bp

coincides with the sequence of the continental specimens. The provided amino acid sequence resulted in two haplotypes for the *tRNA*^{Phe} gene.

B. Specimens from museum collections and donations

I. Specimen 35928 from Trinidad

The identity to the subspecies level of specimen 35928 is questionable. The reason is the high number of nucleotide differences presented in the sequenced genes and region when compared to the rest of the samples used in this investigation. When this specimen was collected, it was not identified to the subspecies level. Museum guidelines designated that identity of the subspecies must be assumed by location. Further inquires on the confirmation of this specimen lead to the specimen collector skeleton data. These measurements coincide with *M. bonariensis minimus* detailed in Lowther and Post (1999), but the measurements also converged with other subspecies such as *M. bonariensis bonariensis* and *M. bonariensis venezuelensis*. Both subspecies are the most proximate to *M. bonariensis minimus's* original range. Yet, due to the locality of the collection, *M. bonariensis venezuelensis* would seem the most probable. This is because the Pacaraima Mountain range ought to have served as a geographical barrier between *M. bonariensis bonariensis* and the northern coast of Venezuela. The highest elevation within the Pacaraima mountain range is found at Mount Roraima with 2,810 m (Braun *et al.* 2003), and the SHCO has not been documented over 2,000 m in altitude (Lowther and Post 1999). Due to the high degree of divergence presented by this specimen, it is important to consider the possibility that it might not be *M. bonariensis minimus*. In addition, a possibility exists that 359228 could be a hybrid specimen of *M. bonariensis minimus* and

either *M. bonariensis bonariensis* or *M. bonariensis venezuelensis* with the maternal lineage originating from either of the latter.

II. Specimens 475F and 475M from Kourou, French Guiana

Specimens from French Guiana possess identical sequences for the five genes and control region. These samples are remarkably similar to those obtained in the expanded area. This finding might be interpreted as individuals introduced to the Greater Antilles originated from continental source, perhaps French Guiana.

III. Specimen 389288 from Roraima, Brazil

This specimen also resembles much of the expanded range sequences and the sequences from French Guiana. The Brazilian specimen, however, is very similar to that of the French Guiana specimens, differing only by two nucleotides in the *cyt b* and *NADH2* genes. Its genetic distance from the expanded range is equal to that of French Guiana and it is thus possible that genetic variation within the subspecies is small and rather homogenously distributed across its original range. In that scenario, the origin of the expanded range haplotype could be any place within the original range. Only the nucleotide difference in the *NADH2* gene confers a nonsynonymous change.

C. Relevance of mtDNA

A recent meta-analysis reports that in contrast to what was previously thought, nuclear DNA is a better indicator of species abundance and fitness than mtDNA (Bazin *et al.* 2006). Bazin and other collaborators argue that mtDNA is not an effective indicator of either population size or species fitness, but rather corresponds to recently selected, well-

adapted haplotypes. This finding suggests the necessity to investigate the composition of nuclear DNA in order to establish with accuracy the genetic diversity of *M. bonariensis minimus*, because even though no differences were found in the expanded range specimens, nucleotide differences may exist in the nuclear genome. In addition, if I apply the findings of this investigation to Bazin's meta-analysis interpretation, it would imply that the low genetic diversity of the SHCO is a result of the well-adapted haplotype to the current environment.

Under neutral expectations, DNA regions exhibiting higher levels of divergence within species will also show higher levels of divergence between species. When this expectation is not met, it is commonly found that the rate of nonsynonymous substitutions over synonymous substitutions is higher between species than within them. This higher rate is attributed to positive selection leading to speciation. However, in our case, this rate is lower between subspecies than within them. This could suggest positive selection within the subspecies, including the expanded range.

Alternatively, the control region may be under stricter selective constraints than coding regions and, thus may not represent sites as neutral well as silent sites within coding regions. It is noteworthy that with respect to Trinidad and among other icterids, divergence within NADH6 is higher than in the control region.

II. Genetic diversity of the Shiny Cowbird

Genetic diversity estimates of the genes and regions in the sampled original and expanded range revealed limited biodiversity in the original range. Table 8 presented the genetic diversity estimates generated in this investigation. *NADH2* genes, for instance, revealed a low value of $\hat{H}$ of 0.6666667.

An average estimate of F_{ST} resulted in 0.86. This estimate indicates that the sampled populations are different and that considerable amount of differentiation between the sampled populations has occurred. This is a remarkable finding because it suggests that the populations in the expanded range and in the original range do not interbreed. Consequently, migration between the original and expanded ranges does not seem to be a common occurrence in M. b. minimus. If migration or a small group of individuals happens to get to the Greater Antilles, these do not breed, and their haplotypes becomes lost. A comparable study on the differentiation between two *Icterus spurius* subspecies indicated an analogous F_{ST} estimate of 0.608 (Baker *et al.* 2003). These concluded that isolation occurred recently within this subspecies, hence, the high differentiating estimate.

Nucleotide diversity is low within the sequenced genes and regions. *NADH2* followed by *NADH6*, presented the highest rates of nonsynonymous changes. *NADH6* was found with the highest percentage of polymorphic sites within the protein-coding sequences followed by *NADH2*. This reflects that these genes provide more information in phylogenetic studies than other protein-coding genes such as *cyt b* and non-coding genes and regions such as the tRNA gene the control region. An average of 0.00043 resulted for the nucleotidic diversity of the SHCO, and *K* resulted with 0.00141 in average. Neutral theory predicts that these estimates should be equal, thus the difference between these estimates reflects the population expansion suffered or positive selection in the SHCO (Forster *et al.* 1996).

The genetic estimates, in conclusion, indicate that the population in the expanded range is differentiated and that there is no or very low gene flow between the expanded and original range populations. Thus, it indicates that the subspecies does not migrate and that the expanded populations once founded have not been supplemented with other migrating individuals. Low heterozygosity, high differentiation index, and low genetic diversity suggest that Wiley's theory of "island hopping" is the least probable.

III. Phylogenetic trees

MtDNA sequence data is useful for phylogenetic studies, because of its non-recombinatorial mode of inheritance and its conservative evolution with regard to gene order in its protein-coding genes, conservative amino acids replacements, and occurrence of insertion and deletions (Moore and DeFilipis 1997). The trees generated in this investigation corroborate the distance within the *Molothrus* species and within the *M. bonariensis* subspecies (Figs. 13-19).

The *cyt b* and *NADH2* genes have been used in other organism to resolve phylogeny to the subspecies level (Roy 1997, Franck *et al.* 2000, Baker *et al.* 2003, Saarma and Tumanov 2006). *NADH6*, however, has been rarely used as a phylogenetic gene and to my knowledge no investigation has corroborated the resolution of the *NADH6* gene to the subspecies level. On the other hand, the high nonsynonymous rate presented by the *NADH6* gene in the SHCO might be an indicator a good quality gene for this sort. In addition, neither of these tRNA genes or control region has been used in birds for phylogeny purposes. More evidence to the point, the limited haplotypes found will not allow confident phylogenetic differentiation between the sampled specimens.

The generated additive trees by this investigation are in accordance to other publications (Lanyon and Omland 1999, Johnson and Lanyon 1999), except the *Agelaioides badius* phylogeny, which was not included. All *M. bonariensis* specimens reflected a minimum bootstrap value of 95 in the phylogenetic trees presented in this investigation. The trees clustered the expanded range specimens with a minimum bootstrap value of 63. In addition, the generated trees clustered the continental specimens in a clade that was divided into the specimens from French Guiana and Brazil. The minimum bootstrap values were 61. An exception to the continental specimens clustering was observed at the *cyt b* tree in Fig. 13, which grouped the specimens from French Guiana on a separate clade and the specimen of Brazil with the expanded range specimens. The

specimen from Trinidad always appeared separate from the *M b. minimus* specimens, but it always emerges within the *M. bonariensis* species taxon group. In Fig. 18 (*NADH2* and *NADH6* tree), it appears as a separate branch from *M. b. occidentalis*. This supports the notion that specimen from Trinidad is most likely a subspecies other than *M. b. minimus* and *M. b. occidentalis*. The trees constructed from the sequences of genes, *cyt b*, *NADH2,* and *NADH6*, edited to incorporate GenBank sequences of lower quality, are presented in Fig. 19a-c. Fig. 19b represents the same additive tree without *Junco hyemalis* sequence, where the division within the *M. bonariensis* specimens is clearly presented. In addition, Fig. 19c was included to visualize the minimum differences between these specimens. Table 10 presents a matrix of the distances of the specimens in Fig. 19 where the average distance between these specimens is 0.0334. Meanwhile, a maximum distance between the *M. b. minimus* specimens of 0.0025 was observed. This also reveals the limited genetic diversity between the sampled specimens of the *M. b. minimus* subspecies.

IV. Theories on the range expansion of the Shiny Cowbird

Results indicate that the introduction of *M. b. minimus* into the Caribbean has caused great reduction in its genetic diversity. Unfortunately, this reduction has not limited the success of the species. Presumably, due to the advantageous generalist strategy, *M. b. minimus* exploits a range of new victims and hosts species.

It was hypothesized that the original range would hold a larger number of haplotypes for the genes and region investigated. This investigation found; one polymorphism for $tRNA^{Glu}$ genes, two for control region and $tRNA^{Phe}$ genes, three in the *cyt b* and *NADH6* genes, and four in the *NADH2* genes, while in the expanded range only one haplotype was found in the same genes and the control region. This evidence supports the idea of a founder effect in its range expansion into the Caribbean and the North American continent. Thus, the SHCO may represent a case of genetic drift caused by the emigration of individuals from the original range into the expanded range.

The close phylogenetic relationship between the YSBL and the SHCO has eased its reproduction in Puerto Rico. As in other islands, the host species decayed to the point where the SHCO population was no longer able to benefit, and the population of SHCO was too big to be supported by the local avifauna or the resources, such as are food, water, and shelter. The non-specificity of the female SHCO in its laying season brought the parasitism of other species, including the Red-legged Thrush, Zenaida and Common Ground Doves, and the Nutmeg and Bronzed Mannikins with previously documented zero or near to zero parasitism rates in Puerto Rico.

Island-extinction has been documented for the SHCO in the Lesser Antilles. That is the case in Guadalupe, Marie Galante, and Antigua (Lowther and Post 1999). In addition, the range expansion culminated early in the 1990's in the North American continent. Since then, the SHCO has not been documented as spreading more into the continent; in fact, some bird-watchers have noted that SHCO are found locally and in small numbers in groups of mixed species (Bowman *pers.*

com.). This is an interesting observation because it might indicate that the SHCO sister species *M. ater*, native in to North American continent has limited the establishment of these cowbirds. These limitations, for instance, may be aggressive behavior in the competition for quality habitat and hosts. The effects that the SHCO has had in the North American continent have not been investigated. This might reveal a limitation of the SHCO capacity to expand and compete against other brood parasites. Perhaps this is due to the difficulty in locating a population and then distinguishing it from *M. ater*. A study effectuated in Florida could not demonstrate successful breeding of the SHCO because of the difficulty of distinguishing SHCO fledglings from *M. ater* (Post *et al.* 1993). Further investigation is needed.

Models for the range expansions of the SHCO are:

A. Bond's hypothesis

Bond explored the putative origin of the SHCO in the West Indies as part of his investigation for his well-known book: Birds of the West Indies. On following publications, Bond noted that a male and a female SHCO were trapped in Barbados and were released in St. Croix in 1934. Shortly after, the male was collected and donated to the U. S. National Museum, currently known as the Smithsonian Institute (Bond 1956).

The genetic patterns shown by my investigation could be explained if these birds released in St. Croix caused the range expansion of the SHCO in the West Indies. Currently, I am in the process of requesting the male SHCO collected in St. Croix. Information obtained from this specimen might present direct evidence on Bond's hypothesis. Nonetheless, because the female is who provides the mitochondrial germ line, the male will not necessarily provide the desired information.

B. The taxon cycling model

A model proposed to explain the localized extinction and speciation of species is that of taxon cycling. Wilson (1959, 1961) described taxon cycling as sequential phases of expansion and contraction of the ranges of species associated usually with shifts in ecological distribution and adaptations to changing ecological relationships through the cycle. Ricklefs and Cox (1972) applied this model to West Indian birds. They divided the model into four characteristic stages through which a species passes in evolutionary time.

I- Species with widespread or continuous distribution through the islands with little or no subspecies.

II- Species that are generally widespread throughout the islands but that show considerable differentiation of populations into subspecies, gaps may appear across its distribution in small islands.

III- Species with fragmented and reduced distributions and well-differentiated populations.

IV- Species endemic to a single island.

Ricklefs and Cox (1972) indicate that the SHCO coincides with the taxon-cycling model in the first stage. Forty years have elapsed since the collection of the data that elucidated this observation, and conceivably the SHCO is advancing forward to the second stage. Evidence that suggest the advancement of the SHCO is that of the extinction of populations in small islands as is the case of Antigua, Guadalupe, and Marie Galante (Lowther and Post 1999). Ortega (1998) indicated that differentiation in the morphology was evident between SHCO individuals in the populations in Puerto Rico and St. Lucia. Morphological differences presented were in color intensity in the females and size (Ortega *pers. com.*). Perhaps this observation represents the initiation of differences within the diverse

population in the original range and that of the novel and limited in diversity of the expanded range.

C. Natural selection

Mutations are important in evolution because they generate diversity. The existence of only one haplotype for all the analyzed genes and regions of the specimens in the expanded region reflects a limited diversity. If the population present in the expanded range was based on a small number of individuals, it is conceivable that natural selection has acted on the population level on species and has favored the most suited individuals. On the contrary, if the population at the expanded range was based on a pair of individual natural selection, most likely, has not influenced the novel population. As a result, the possibility of an inbreeding depression cannot be ruled out for the subspecies. Even so, this investigation was not carried out to explore inbreeding coefficients.

Although the divergence of this subspecies is clear, it does not necessarily imply that natural selection has occurred. I found that K is larger than π through the mtDNA sequences and this is a signature of positive selection. However, it is also a signature of population expansion and there is no doubt that this has occurred in this subspecies (McDonald and Kreitman 1991). As stated above, the ratio of non-synonymous over synonymous mutations is higher between the expanded and original ranges than between Trinidad and the rest of the samples, and this is consistent with expectations of positive selection, but the sample size is too small to make a strong statement. Population expansion usually leads to a reduction of divergence, and the SHCO has been able to expand its range due to anthropogenic factors. The SHCO is, consequently, evidence of the detrimental effects caused by alterations in the environment.

CONCLUSIONS

- The mitochondrial gene order in the amplified fragments and sequenced genes of the SHCO agrees with the order stipulated by Desjardins and Morais (1990).

- The nucleotide compositions of the sequences generated are in accordance to other avian studies.

- Sequence from the five genes and the control regions in all 34 SHCO specimens of the expanded range are identical, thus, presented only one haplotype.
    - No genetic variation was discerned within the SHCO samples collected in 1987 and those collected in 2004-2005 in Puerto Rico.

    - No genetic variation was discerned within the SHCO samples collected in the states of Florida and Louisiana in the USA.

- Sequence from the five genes and the control regions in three sampled specimens of the original range present two haplotypes, one for each country.
    - Continental specimens differ by two nucleotide polymorphisms.

    - Continental specimens differ from expanded range specimens by five nucleotide polymorphisms.

- Sequenced *NADH6* genes reflect a higher polymorphism of 2.5% than other genes or regions.

- Data is consistence with a one female origin for the entire expanded range population; hence, the current population is based on a founder event.

- The SHCO, because of its detailed range expansion across the Caribbean and North American continent, and hypothesized introduction pattern might serve as a model for mutations on genetic investigations.

RECOMMENDATIONS

Future research can integrate other molecular techniques of estimating genetic diversity within the subspecies, such as, AFLP, microsatellites, and the sequencing of nuclear genes from the sampled SHCO populations in the expanded and original range. Further investigations with other techniques, may provide additional information differentiation of these specimens, and on the migration pattern realized by the SHCO during the last century. In addition, the genetic diversity estimated through other techniques could serve as a comparison for the genetic diversity estimates obtained in this investigation. Perhaps an interesting area would be to investigate if an inbreeding depression can be measured in the expanded range individuals. Also, the validity of the various NADH subunits and of other genes in the mtDNA as reliable phylogenetic data might be useful to explore; therefore, I highly recommend the complete sequencing of the mtDNA in these specimens.

A comparison between SHCOs of the Lesser Antilles to those obtained in this investigation in the expanded range should be investigated to confirm Bond's thoughts of two distinct origins of dispersal of the SHCO. Further sequencing of other subspecies is highly recommended. Specially, of that of other subspecies like, *M. b. venezuelensis* and *M. b. bonariensis,* in order to assess specimen 35928 of Trinidad.

Finally, an intriguing pattern has been observed in the expansion of *M. ater* range. After the SHCO reached Canada and Oklahoma in early 1990, its advance stopped. Perhaps there is no sustainable coexistence between the native *M. ater* and the introduced *M. b.minimus* in the North American continent. The coexistence of these sister taxa should be investigated.

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APPENDIX

I. Parasitized Yellow-shouldered Blackbird (*Agelaius xanthomus*) nest with a SHCO egg (left).

II. Parasitized Yellow Warbler (*Dendroica petechia*) nest with a SHCO egg (top left).