# The New World diversification and origins of the Buckeye butterflies (genus *Junonia*, Nymphalidae: Nymphalini)

by

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**Abstract**

The New World buckeye butterflies (genus *Junonia*) are valuable experimental model organisms, but the taxonomy of this group has been problematic and contentious. I have clarified the taxonomy of the *Junonia* species in the Western Hemisphere using molecular and morphological data from contemporary and museum collections, with a focus on the South American *Junonia* species. To do this, I have developed and validated a restriction-digest based mode of mitochondrial genotyping for use with both contemporary and historical specimens, which may be used once species have been identified based on morphology. An improved taxonomy will encourage and support further comparative biology research in *Junonia*. I have also tested the hypothesis that *Junonia* populations in the New World comprise a ring species. I falsified one of the predictions of the ring species hypothesis by detecting no discontinuity in gene flow based on mitochondrial genotype data in the proposed area of overlap between the ends of the ring. This region of overlap is where a true ring species would exhibit reproductive isolation. To clarify the relationships among *Junonia* species and address the issue of the origins of the New World *Junonia*, an extensive molecular phylogenetic analysis was completed using both full mitogenome and nuclear rRNA repeat sequences. Like previous molecular phylogenies based on much smaller DNA fragment data sets, the species level relationships of the New World *Junonia* were inconclusive because they were obscured by recent divergence of lineages and gene flow between species. Based on full mitogenome and rRNA repeat phylogenies, I was able to add additional support for a trans-Pacific route of Asian species responsible for the New World *Junonia* diversification, though some data suggest that genetic contributions from trans-Atlantic migrants from other *Junonia* lineages is also possible. The New World *Junonia* may be an example of lineage hybridization contributing to rapid diversification and adaptive radiation.

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# Chapter 1: Introduction

## ***Overview***

This dissertation will focus on the diversification and origins of the New World buckeye butterflies (genus *Junonia*). I will investigate the geographic variation in the New World *Junonia* and reconstruct the origins and the evolutionary history of their recent and rapid diversification event using biogeographic data, life histories, and mitochondrial DNA using the DNA barcoding fragment (*Cytochrome oxidase subunit I*), complete mitochondrial genomes (mitogenomes), and nuclear rRNA repeat sequences. This introductory chapter outlines topics and concepts that are necessary background for understanding the experimental chapters of the thesis. These include gene transfer, gene flow, hybridization, adaptation, speciation, species concept, ring species, adaptive radiations, rapid radiations, mitochondrial DNA uses and functions, as well as the utility of the nuclear rRNA repeat. I also introduce the evolutionary and developmental model organism, the buckeye butterflies of genus *Junonia*. The work described in this dissertation validates a genotyping method for mitochondrial haplotyping in historical museum specimens that facilitates documenting patterns of gene flow over space and time. It will demonstrate how one can use museum specimens to reconstruct a recent invasion event of a group and investigate the *Junonia* ring species hypothesis. This brings with it enhanced knowledge of *Junonia* species diversity in Central and South America in this genus, and documents interspecific gene flow. Lastly, I will explore how full mitogenome and nuclear rRNA repeat data can be used to reduce the challenges posed by ambiguities, missing taxa, and polytomies that exist in current phylogenetic reconstructions based on small molecular data sets. I will try to reconstruct the origins and evolutionary history of the recent and rapid diversification of New World buckeye butterflies (genus *Junonia*), and explore the details of this explosive biological radiation, yielding a broader understanding of the processes of speciation and adaptive radiation.

## ***Gene transfer, gene flow, hybridization, and adaptation***

Gene transfer is a widespread phenomenon with important impacts for all living things. Gene transfer is accomplished in two main ways: Vertical gene transfer occurs from parent to offspring and is the predominant mode of inheritance for life on earth. Horizontal/lateral gene transfer occurs between a donor organism and an unrelated recipient organism but can occur between individuals of the same species (Burmeister 2015; Lorenzo-Díaz *et al.* 2017). Vertical gene transfer is most common among unicellular and multicellular organisms and is responsible

for the maintenance of species and population identities (Burmeister 2015; Lorenzo-Díaz *et al.* 2017; Babcock 2020). Horizontal gene transfer is most common in bacteria and is one of main drivers of antibiotic resistance, which is transferred from one bacterial lineage to another (Burmeister 2015; Lorenzo-Diaz *et al.* 2017). The phenomenon of genetic transfer of DNA between organisms is significant as it allows species groups to maintain their identity, it is the basis for the creation of new genes, and it permits researchers to track changes that occur in populations of organisms. Movement of specific genetic information between groups of individuals in populations is known as gene flow (Darwin 1859; Mayr 1963; Slatkin 1987).

Measuring movement of genetic variation between populations is a method that many researchers use to assess the natural state of populations and evaluate the connectivity of among them. These considerations are important for understanding the evolution of species, the process of speciation, and mechanisms of species diversification (Mayr 1963; Cracraft 1983; Remington 1985; Slatkin 1987). Gene transfers between populations can occur in a few ways including, conspecific organisms bringing in new genetic variation from other populations by means of migration, hybridization, and introgression (Mayr 1963; Cracraft 1983; Remington 1985; Schluter 2009). Gene flow occurs at the population level and is the result of the accumulation of gene transfers from individuals within them (Ehrlich and Raven 1969; Slatkin 1987). Researchers can estimate the amount of gene flow that is occurring between populations in order to characterize population dynamics through direct methods (breeding success and dispersal) or indirect methods (allele frequencies, genetic comparisons) (Slatkin 1987). Gene flow is also considered a limiting factor in the evolution of populations because although populations tend to carry genes that are adapted to their local environment, organisms coming from different environments bring with them allelic variation that has been under selection by different environmental conditions. This variation may either be beneficial or detrimental when it is introduced into a recipient population (Ehrlich and Raven 1969; Slatkin 1987).

The degree of gene flow between populations of the same species is to some extent a function their mobility throughout a specific geographic range (Remington 1985; Slatkin 1987). Populations in any geographic range will occupy areas with suitable habitat and individuals from different populations will move between these habitats, and into new suitable ones. Over time, the range of a species will expand and contract (Remington 1985; Slatkin 1987). Species will

tend to expand their ranges to suitable habitats until some sort of barrier stops them from expanding further such as mountain ranges, bodies of water, and deserts. In addition, other barriers that may or may not be easily identified include abundance of resources, predators, competition with other species, or climate (Remington 1985; Slatkin 1987). The overall distribution of populations may be continuous over a geographic area or result in small population patches (Remington 1985; Slatkin 1987). The amount of gene flow that occurs between these populations depends on many biological features of a particular species (Remington 1985; Slatkin 1987). If gene flow is consistent between populations, no large differences in the genetic make-up of populations will come about, but if gene flow does not occur in any particular population over a long period of time, this may promote speciation (Remington 1985; Slatkin 1987).

***Speciation and species concepts***

Speciation can be described as the process in which sexually reproducing organisms completely diverge from other closely related taxa and become their own species unit (Dobzhansky 1940; Cain 1954; Mayr 1963; Maynard Smith 1966; Remington 1985). The mechanism that has been most commonly hypothesized as the main cause of speciation is the cessation of gene flow between reproductively isolated populations (Dobzhansky 1940; Cain 1954; Mayr 1963; Maynard Smith 1966; Slatkin 1987; Wiens 2004; Schluter 2009). This could happen in a number of different ways including a physical barrier that ultimately geographically isolates populations, the exploitation of new niches or resources by individuals in a population, or changes in genes that change behaviour, morphology, or physiological processes that no longer allow for populations to successfully reproduce (Dobzhansky 1940; Mayr 1942; Cain 1954; Mayr 1963; Maynard Smith 1966; Remington 1985; Slatkin 1987; Schluter 2009). The process is considered to be irreversible because when and if reproductively isolated populations come back into contact, the two diverged species are either no longer able to reproduce, or viable offspring are not produced (Dobzhansky 1940; Mayr 1963; Maynard Smith 1966; Remington 1985; Slatkin 1987).

There are four common speciation models that attempt to explain how different populations may become reproductively isolated: sympatric speciation, allopatric speciation, peripatric speciation and parapatric speciation (Dobzhansky 1940; Cain 1954; Mayr 1963; Maynard Smith

1966; Cracraft 1983; Remington 1985; Slatkin 1987; de Queiroz 2007; Aldhebiani 2018). Although all four of these speciation models are important, I will focus on sympatric and allopatric speciation in my dissertation, as these mechanisms will be of importance in the discussion of ring species (below, Chapter 4).

Allopatric speciation occurs when populations of organism become geographically isolated (Dobzhansky 1940; Maynard Smith 1966; Wiens 2004). When a geographical feature physically keeps one population separated from another for an extensive period, these populations accumulate enough differences between them (either physiological, morphological or behavioural), so that if they come back into contact, interbreeding would not occur and at this point the two populations are considered separate lineages (Dobzhansky 1940; Mayr 1963; Maynard Smith 1966). Sympatric speciation is the process by which speciation occurs between populations within the same geographic area or habitat (Dobzhansky 1940; Maynard Smith 1966; Slatkin 1987). Isolation occurs by physiological or behavioural differences accumulating between the populations, such as changes in suitable habitat, resource availability, competition, and species interactions such as mating displays or morphological characteristics (Dobzhansky 1940; Mayr 1942; Maynard Smith 1966; Wiens 2004; Schluter 2009). The commonality between concepts in speciation is reproductive isolation without gene flow. If allopatric populations do come into contact at a later time, the geographic regions where this occurs is considered a secondary contact zone.

Secondary contact zones are areas where populations that have not had contact for a period of time come into contact once again under natural conditions (Remington 1985; Slatkin 1987; Durand *et al.* 2009). Theoretically, if enough time has elapsed and reproductive isolation mechanisms have developed, hybridization would not occur, and these two populations would remain two distinct species. This theory although sound, is not usually what is observed in nature. Many examples of hybridization events between similar species are apparent in nature and where these events happen are considered hybrid zones (Remington 1985; Slatkin 1987; Mallet *et al.* 2007; Keller *et al.* 2013; Meier *et al.* 2017; Lalonde *et al.* 2018; Lamichhaney *et al.* 2018; Lalonde and Marcus 2019a). Examples have been characterized in vertebrate (Turner and Harr 2014; Scordato *et al.* 2017; Lamichhaney *et al.* 2018), invertebrate (Hafernik 1982; Corrêa *et al.* 2019; Lalonde and Marcus 2019a), and plant populations (Rieseberg and Carney 1998;

Abbott 2017). Hybridization has also been suggested as a means of increasing species diversity of groups of organisms and another means of species creation (Barton 2001; Mooney and Cleland 2001; Mallet 2007; Abbott *et al.* 2013), which will be discussed below in the discussion of rapid radiations of species.

Species are considered to be the smallest group of individuals that occur naturally and can be reliably diagnosed as being the same (Mayr 1963; Cracraft 1989; de Queiroz 2005; de Queiroz 2007). Species are considered to be the main unit of comparison when considering all areas of biological study, but especially in the case of systematics and evolution (Cracraft 1983; Cracraft 1989; de Queiroz 2005). Being able to delimit species is of particular importance because it allows researchers to distinguish distinct lineages of organisms and to determine how species are related (Mayr 1942; Mayr 1963; Cracraft 1983; Remington 1985; Slatkin 1987; Cracraft 1989). Although the classification of species is important, the concept of how a species should be categorized is still debated. To date, there have been at least 27 different proposed definitions for how species should be designated taxonomically and the strengths and weaknesses of each one have been reviewed extensively (eg. See (Cracraft 1983; de Queiroz 2005; de Queiroz 2007; Zachos 2016; Aldhebiani 2018)). I will focus on the three most used and discussed species concepts: The Biological Species Concept, Phylogenetic Species Concept, and the Morphological Species Concept.

The Biological Species Concept is one of the most well-known species concepts whose definition was described long before it was formally named by Mayr in 1982. The main focus is on speciation based on reproductive isolation (Dobzhansky 1940; Mayr 1942; Cain 1954; Mayr 1963; Mayr 1982), as described above in discussions of allopatric speciation. Species are defined based on their ability to interbreed within natural populations but do not interbreed with other groups, so are reproductively isolated (Dobzhansky 1940; Mayr 1942; Mayr 1963; Maynard Smith 1966; Mayr 1969; Mayr 1982; Cracraft 1983). Although this concept is straight forward in theory, when considering sympatric populations, the classification of organisms on this basis poses problems in number of different situations. For example, when comparing allopatric populations, or when hybridization events occur naturally in nature, the Biological Species Concept tends to lump taxa that may not be closely related evolutionarily but retain the ability to produce fertile offspring.  At the same time, the Biological Species Concept can exclude taxa

that may be closely related evolutionarily, but which are no longer reproductively compatible because of changes in phenology, geographic isolation, or *Wolbachia* infection. This makes taxonomic classification very difficult because the Biological Species Concept uses reproductive isolation as the sole criterion for defining species and does not take any other biological processes into consideration (Dobzhansky 1940; Mayr 1942; Mayr 1963; Mayr 1969; Mayr 1982; Cracraft 1983; Cracraft 1989; Braby *et al.* 2012).

The Phylogenetic Species Concept was proposed by Cracraft in 1983 as an alternative to the Biological Species Concept that takes systematics and phylogenetic relationships into consideration. Using this concept, a species is defined as the smallest group of related individuals that are diagnosable based on similar character states and shared ancestry (Cracraft 1983). It offers advantages over the Biological Species Concept because it allows for hybridization between species, as reproductive isolation is not a requirement, nor does it rely on species being restricted to a specific geographic locality (Cracraft 1983). This concept also has limitations as it does not take any taxonomic rank into consideration, it may lead to over representation in species diversity, and resulting in over splitting of populations into smaller taxa, creating more species than might be defined by reproductive isolation or morphological characteristics (Mallet 1995; Moritz 2002; Isaac *et al.* 2004; Brooks and Helgen 2010).

The Morphological Species Concept focuses on species delimitation based on morphological characteristics and is the basis for most taxonomic species designations in use today (Cain 1954; Mayr 1969; Mayr 1982; Cracraft 1983; Wiens 2004; de Queiroz 2005; de Queiroz 2007; Zachos 2016; Aldhebiani 2018). This is because it is often not practical to test representatives of every possible new species for reproductive isolation and/or monophyly from related species, and it may be entirely impossible for rare, asexual, or fossil organisms (Bock, 2004). Yet, many limitations exist with using solely morphological characteristics as a basis for delimitation of species. Many populations display phenotypic variation which may lead to incorrect identifications. Morphologically cryptic taxa appear to be very similar morphologically but are different genetically and are not accounted for by the Morphological Species Concept and instead are grouped together within a single species (Knowlton 1993; Kozak *et al.* 2006; De Barron and Ahmed 2011). Finally, distinguishing species by morphology tends to be reliable for certain life stages (for example, the adult stage), but morphological characteristics change during

different life stages, and even between sexes (Mayr 1942; Mayr 1963; Mayr 1969; Cracraft 1983; Cracraft 1989; Mallet 1995; de Queiroz 2005; de Queiroz 2007; Zachos 2016; Aldhebiani 2018).

Based on the analysis of all available species concepts currently proposed, I chose to use a variation of the Biological Species Concept called the Isolation Species Concept to delimit species in all research chapters of this dissertation. The Isolation Species Concept focuses on intrinsic reproductive isolation factors rather than geographic isolation and defines species as systems of populations where genetic exchange between them is either limited or prevented by either one or more isolating mechanisms (not solely extrinsic factors as defined by the Biological species concept) (Mayr 1942; Dobzhansky 1970; Templeton 1989). The Isolation Species Concept does not require populations to be geographically isolated to be reproductively isolated as defined by the Biological Species Concept, in addition it allows for intrinsic factors to serve as the cause for reproductive isolation and is not reliant on solely extrinsic factors being barriers to reproduction (Dobzhansky 1940; Mayr 1942; Mayr 1963; Maynard Smith 1966; Mayr 1969; Mayr 1982; Cracraft 1983). Mobile species such as the buckeye butterflies, do not restrict their interactions to individual species within specific geographic locations, which makes the Biological Species Concept difficult to apply to groups such as this. The genus *Junonia* also have some morphologically similar species that display considerable amounts of both geographical and seasonal intraspecific variation (Forbes 1928; Remington 1985; Rountree and Nijhout 1995), and forms that are capable of hybridization and producing fertile offspring (Hafernik 1982; Paulsen 1996), which signifies that reproductive isolation is not complete, and does not allow for the Biological Species Concept to be applied. The Isolation Species Concept allows for individuals in any place at one time to be considered the same species and reproductively isolated, so allows for the flexibility that natural populations demonstrate such as gene flow occurring between populations and, hybridization, making it an ideal concept for mobile organisms.

***Ring species and how they can be used as a model for speciation***

Ring species are of particular importance in biological study as they have been described as a way to help scientists understand the processes of speciation, the creation of new species, how organisms evolve through time, and how biodiversity is produced (Mayr 1942; Cain 1954; Irwin

*et al.* 2001; Blackmon and Demuth 2012; Martins *et al.* 2013). A ring species can be characterized as a transitional state in the process of speciation that was originally proposed by Mayr (1942), but the term ring species was coined by Cain (1954). Ring species are thought to occur where organisms within a taxon have a ring-shaped distribution around a region of unsuitable habitat, where genetic exchange occurs as a function of distance at all points of the distribution with the exception of the overlapping ends of the ring where there is no gene flow between extreme forms (Cain 1954; Joseph *et al.* 2008; Blackmon and Demuth 2012; Gemmell and Marcus 2015). It can be characterized as when a vicariance event (separation of a population due to a geographic barrier) occurs, and interrupts gene flow around the ring, which represents the transition from a single lineage to two distinct lineages (Mayr 1942; Cain 1954). Ring species formation is a process where geographic space can serve as a surrogate of time: the process of population divergence begins in a specific geographic location (e.g. at the top of the ring or the point of origin). From the point of origin, the populations disperse to suitable habitats on either side of an area of unsuitable habitat allowing for allopatric speciation to take place. Finally, the 2 lineages meet at the bottom of the ring after a period of time, but gene flow does not take place between the two lineages, signifying that reproductive isolation has taken place satisfying the requirement of reproductive isolation of the Biological Species Concept (Mayr 1942; Cain 1954; Mayr 1963; Blackmon and Demuth 2012).

Many characteristics make ring species particularly interesting because they allow researchers to trace microevolutionary changes between populations throughout a geographic range. The ring can serve as a snapshot into the history of a particular taxon's formation, with continued gene flow between peripheral populations demonstrating that gene flow can still occur between populations through a geographic range, which promotes differentiation and not homogenization of populations (Mayr 1942; Cain 1954; Mayr 1963; Irwin *et al.* 2001; Blackmon and Demuth 2012). It is necessary to recognize that ring species are unique as they are an edge case in the Biological Species Concept, that defines species based solely on reproductive isolation. Depending on which populations are selected to be compared for reproductive compatibility within the ring, each may yield different outcomes placing them either as the same or different species. Taxonomists who have encountered either partially or completely uncharacterized ring species, would assign populations to one or more species or subspecies, depending on what information related to reproductive isolation was available, and to what

degree of morphological differentiation that was observed between populations. Consequently, the populations that make up a proposed ring species may be inconsistent in taxonomic rankings, with varying numbers of species, subspecies, or a combination of both, depending on the taxonomic naming conventions, traditions, and taxonomic history for that particular set of biological entities. Due to these aforementioned reasons, the concept of a genus as ring species is possible because the concept does not pertain to specific taxonomic entities within groups, but solely relates to the patterns of biogeography and reproductive isolation (Kuchta and Wake 2016).

To demonstrate how a genus may be considered a ring species, we will consider the buckeye butterflies (genus *Junonia*) in the New World. The *Junonia* butterflies were originally proposed as a ring species by Forbes in 1928. At that time only four *Junonia* species were thought to occur in the New World. Three of these species, *J. coenia, J. evarete* and *J. genoveva* were described as being highly polymorphic with distinct populations that merged into one another, except for Cuba where reproductive isolation was suggested to occur between *J. coenia* and *J. evare*te (Forbes, 1928). Since 1928, many additional species and subspecies have been described in this group (Brévignon 2008; Neild 2008; Brévignon 2009; Brévignon and Brévignon 2012; Lalonde and Marcus 2019a). Despite the fact that many of the forms have been given full species status based on life histories, biogeographic distributions, morphology, and genetic differences, reproductive isolation has not been established between them (Hafernik 1982). Regardless of the taxonomic rank given to these named taxonomic entities they have biological characteristics and geographical distributions that resemble other well described examples of proposed ring species.

Ring species have been considered a case of evolution in action, as they are speculated to allow researchers to observe gene flow between chains of interbreeding population and the eventual the creation of new species over time. Many possible ring species have been proposed, but with further investigation over the years, most do not meet all of the criteria of a ring species (Forbes 1928; Mayr 1942; Cain 1954; Mayr 1963; Price *et al.* 1997; Highton 1999; Irwin 2000; Irwin *et al.* 2001; Liebers *et al.* 2004; Kozak *et al.* 2006; Joseph *et al.* 2008; Kuchta *et al.* 2009; Sternkopf *et al.* 2010; Blackmon and Demuth 2012; Cacho and Baum 2012; Martins *et al.* 2013; Gemmell and Marcus 2015; Kuchta and Wake 2016; Bouzid *et al.* 2021). All proposed examples display a biogeographical species distribution in the shape of a ring, are composed of

morphologically similar species that occur around this distribution, and a discontinuity where species do not interbreed. Where most proposed ring species fail is regarding the genetic exchange between the chains of interbreeding populations that habituate around the ring. The three most compelling examples of ring species that have been used to test the ring species hypothesis but have failed to meet all criteria are the herring gull complex (Mayr 1963; Liebers *et al.* 2004), the greenish warbler complex (Mayr 1942; Mayr 1963; Price *et al.* 1997; Irwin 2000; Irwin *et al.* 2001), and the *Ensatina* salamander complex from California (Wake and Yanev 1986; Wake *et al.* 1986; Moritz *et al.* 1992; Kuchta *et al.* 2009). All three of these examples will be discussed below to show where each satisfy and fail the criteria used to define and constitute a ring species

The herring gull complex (genus *Larus*) has a ring-shaped distribution centered around the Arctic Ocean (unsuitable habitat), and in Northern Europe a discontinuity exists between morphologically similar species (Mayr 1942; Mayr 1963; Liebers *et al.* 2004; Sternkopf *et al.* 2010). In the area of the discontinuity where the sympatric populations are present, it was found based on molecular data that both species have different biogeographic histories and the ancestors of these two populations did not even occupy the same glacial refugia, therefore gene flow is not continuous around the ring from a single source population (Liebers *et al.* 2004; Sternkopf *et al.* 2010). Although not a ring species, it has been suggested that the genus *Laurus* is relatively young, and that not enough time may have passed for the genetic signals needed to quantify gene flow to be apparent (Liebers *et al.* 2004; Sternkopf *et al.* 2010).

Second, the biogeographic distributions of the salamanders from the genus *Ensatina* from California, USA are interesting as they display a morphological gradient from a northern freely interbreeding population in Northern California, dispersed around the unsuitable habitat in the Central Valley, that occurs between 2 different mountain ranges, and in Southern California sympatric populations that do not interbreed (Wake and Yanev 1986; Wake *et al.* 1986; Moritz *et al.* 1992; Kuchta *et al.* 2009; Blackmon and Demuth 2012; Kuchta and Wake 2016). Molecular data has shown that gene flow around the ring occurs in patches and is not continuous in nature suggesting distinct lineages. Further, a secondary large discontinuity in both gene flow and morphology occurs in the Northeastern portion of the range in a region called Lassen Peak (Wake *et al.* 1986; Highton 1999; Kuchta *et al.* 2009; Blackmon and Demuth 2012). Like the

previous example, the salamanders fail the criterion of having continuous gene flow, which is required to be classified as a ring species (Mayr 1942; Cain 1954; Mayr 1963).

What is widely thought to be the best (yet, still imperfect) example of a ring species are the greenish warblers (genus *Phyloosopus*) (Mayr 1942; Mayr 1963; Irwin 2000; Irwin *et al.* 2001; Irwin 2002; Irwin *et al.* 2005). The biogeographic distributions of the warblers occur around the Himalayas (unsuitable habitat) and have sympatric populations that occur in Siberia (Price *et al.* 1997; Irwin 2000; Irwin *et al.* 2001; Irwin 2002). Interbreeding does not occur in Siberia based on song variation as the two species do not recognize the song of other populations, and around the ring the song complexity varies from less complex in the south to more complex in the northern portions of the range (Irwin 2000). Although both species lineages come from a single source population (Price *et al.* 1997), a large additional discontinuity in gene flow occurs in China due to anthropogenic deforestation and has existed for an extensive period of time (Irwin 2002). So, this example also fails on the criterion of continuous gene flow.

Although no examples have been identified that satisfy all of the criteria required for a ring species, the concept of ring species and the imperfect examples that exist are still very useful as they allow researchers to explore how speciation can occur by focusing on allopatric and sympatric models, and how isolation by distance can be an effective way to explain the divergence of species (Dobzhansky 1940; Mayr 1942; Cain 1954; Mayr 1963; Maynard Smith 1966). Ring species can also be used to explore questions related to convergent evolution (independent evolution of similar forms of organisms, processes, and traits), because similar habitats and resources must exist similarly on each side of the ring (Arendt and Reznick 2008; Powell 2012).

The buckeye butterflies (genus *Junonia*) in the New World were proposed as a ring species by Forbes (1928) based on morphological characteristics and an apparent discontinuity in forms in Cuba. The biogeographic distributions of *Junonia* occur in a ring-shaped distribution, with the unsuitable habitat being the Caribbean Sea and Gulf of Mexico (Forbes 1928; Gemmell *et al.* 2014; Gemmell and Marcus 2015). The apparent discontinuity originally proposed in Cuba (Forbes 1928) was not supported based on molecular data that shows that gene flow is ongoing between the forms in this region (Gemmell and Marcus 2015; Lalonde *et al.* 2018; Lalonde and Marcus 2019b). However, based on additional molecular data, a second possible disruption in

gene flow was suggested in Panama and northern South America, a region where species are not well characterized in this genus (Gemmell and Marcus 2015). Exploring the proposed transition zone in Panama and northern South America, will allow for the characterization of species, create biogeographic distributions of the species in the region, and allow for testing if a discontinuity occurs in relatively recently diverged genus (Chapter 4).

***Adaptive and rapid radiations***

Adaptation is the process by which an organism responds to changing environmental conditions (Dobzhansky 1940; Axelrod 1962; West-Eberhard 1986). Understanding how these changes occur, the diversification of traits throughout groups, and the evolutionary history of groups of organisms are important topics in the field of biology (Cracraft 1989; Arendt and Reznick 2008; Pfennig *et al.* 2010; Gardner 2017). Phenotypic plasticity is one of the means by which organisms have the ability to respond to changes in environmental conditions (Stearns 1989; West-Eberhard 1989; Agrawal 2001; Pfennig *et al.* 2010). Phenotypic plasticity is the capacity of an organism to use their existing genes to produce different phenotypic responses. The phenotypic changes in response to a changing environment can include physiological, morphological, or behavioural changes that increase an organism's chance of survival (Ford 1966; Stearns 1989; West-Eberhard 1989; Agrawal 2001; Pfennig *et al.* 2010). Because these adaptive responses can change many characteristics of an organism, adaptation has been suggested as a means of species diversification, and subsequently a method of speciation (Stearns 1989; West-Eberhard 1989; Agrawal 2001; Fusco and Minelli 2010; Pfennig *et al.* 2010).

Adaptive radiation is the ability of a group of organisms from the same taxon to spread into many different environments and habitats (Axelrod 1962). This phenomenon is commonly explained in relation to rapid range expansions of species, after periods of restricted range due to some abiotic factor such as glaciation (Cristescu 2015; Nichols *et al.* 2015). Rapid expansion of ranges of species may allow for previously allopatric populations to become sympatric again. If secondary contact and hybridization occurs between these populations it can be a means of a quick explosion of species, increasing species diversity in groups very rapidly (Seehausen 2004; Heliconius Genome Consortium 2012; Nichols *et al.* 2015). Rapid radiations of species is one mechanism that has been used to explain poorly resolved groups of organisms in phylogenetic

reconstructions, this is especially true for recently diverged groups, where differences in species (all characters) may not have had enough time for morphological, physiological or genetic differences to occur (Nichols 2001; Rosenberg 2002; Whitfield and Lockhart 2007; Giarla and Esselstyn 2015). If the divergence is sufficiently rapid, many lineages can share characteristics resulting from incomplete lineage sorting (Nichols 2001; Rosenberg 2002; Whitfield and Lockhart 2007). When working with molecular phylogenetic reconstructions of recently diverged species which tend to be poorly resolved, more robust phylogenetic reconstructions can be obtained by the inclusion of 2 or more distantly related outgroup taxa than can be obtained from analyses using a single outgroup species (Shavit *et al.* 2007). Rapid diversification and adaptation have been well characterized in the haplochromine cichlids of Lake Malawi and are a great example how secondary contact and hybridization can cause rapid radiation and speciation (Greenwood 1984; Moran *et al.* 1994; Stiassny 1999; Danley *et al.* 2000; Nichols *et al.* 2015). The buckeye butterflies are suggested to have diverged relatively recently and rapidly in the New World, causing phylogenetic reconstructions of species groups to be poorly resolved (Kodandaramaiah and Wahlberg 2007; Pfeiler *et al.* 2012; Gemmell *et al.* 2014; Gemmell and Marcus 2015; Peters and Marcus 2017; Cong *et al.* 2020). Using multiple outgroup species in phylogenetic reconstructions may help in disseminating the New World *Junonia* species (Chapter 5).

***Mitochondrial DNA functions and uses and the utility of the nuclear rRNA repeat***

Deoxyribonucleic Acid (DNA) is the hereditary carrier of genetic information in the cells of organisms (Avery *et al.* 1944). DNA is passed onto to offspring through reproduction either by self-fertilization, cloning, or by sexual reproduction (through genetic exchange between individuals) (Weismann 1890; Hillis 2007). Mitochondrial DNA (mtDNA) is found solely with the mitochondrion and has an asexual means of passing on its genetic information, directly from mother to offspring without recombination (Wilson *et al.* 1985; Saccone *et al.* 1999). The mitochondrion is the primary eukaryotic energy releasing organelle derived originally from a symbiotic relationship between a primitive aerobic prokaryotic and the eukaryotic cell, therefore having a different and distinct genome from the nuclear genome (Saccone *et al.* 1999; Saccone *et al.* 2000; Godfrey-Smith 2015). Due to its biological function to maintain cellular processes in

the cell, it is important and considered to be highly conserved between all animal species (Brown *et al.* 1979; Vawter and Brown 1986).

The mitochondrion is an important organelle as it is responsible for the energy production of the cell. Energy is produced from the breakdown of carbohydrates, and it occurs in high copy number, has a haploid genome that contains all of the genetic information necessary for this to occur (Nass 1969; Brown *et al.* 1979; Wilson *et al.* 1985; Boore 1999; Saccone *et al.* 1999; Al-Nakeeb *et al.* 2017). In animals, the mtDNA genome is a circular molecule that varies in size from ~13–20 kilobases, and gene order is conserved in closely related species (Boore 1999; Saccone *et al.* 1999). It lacks introns and exons, with the genes often overlapped or separated by only a few nucleotides, and is composed of 13 protein-coding genes, 2 ribosomal RNAs (rRNA), and 22 transfer RNAs, and a control region that controls the initiation of replication and transcription (de la Cruz *et al.* 1984; Boore 1999; Saccone *et al.* 1999; Saccone *et al.* 2000). Mitochondrial DNA evolves much faster than nuclear DNA because the mutation rate is higher, evolutionary changes occur rapidly, and base substitutions and indels are rare (Brown *et al.* 1979; Wilson *et al.* 1985; Boore 1999).

Mitochondrial DNA sequences can be easily recovered from organisms, as it is simple to amplify and sequence using polymerase chain reaction (PCR) or using whole genome sequencing (WGS) (Saccone *et al.* 1999; Al-Nakeeb *et al.* 2017). The most commonly used mitochondrial gene for addressing evolutionary questions is Cytochrome c oxidase subunit I (*COI*), also known as the DNA barcoding gene, because it allows for researchers to detect small genetic differences (genotyping) which often have a useful strong phylogenetic signal (Boore 1999; Hebert *et al.* 2003b; Hebert *et al.* 2003a; DeSalle *et al.* 2005; Janzen *et al.* 2005; Ratnasingham and Hebert 2007). Using DNA barcoding has proven useful for species delimitation and resolving ambiguities in the taxonomy of some groups, based on the unique genotypes of many species (Meusnier *et al.* 2008; Zink and Barrowclough 2008; De Barron and Ahmed 2011; Price *et al.* 2015). Traditionally, part of the *COI* gene of the mitochondrial DNA (658 bp sequence that is considered species specific for some organisms) is used for DNA barcoding in animals as the primer binding sequences are considered to highly conserved among organisms (Folmer *et al.* 1994; Hebert *et al.* 2003b; Hebert *et al.* 2003a; Hajibabaei *et al.* 2006). Although in some groups that have diversified very recently, like the butterfly genus *Junonia*, the

*COI* gene has proven to be difficult to use (Janzen *et al.* 2005; Pfeiler *et al.* 2012; Borchers and Marcus 2014; Gemmell *et al.* 2014; Gemmell and Marcus 2015). This has been attributed to the fact that in addition to its recent origin, hybridization and mitochondrial introgression takes place between many New World *Junonia* species (Hafernik 1982; Borchers and Marcus 2014; Gemmell *et al.* 2014; Lalonde *et al.* 2018; Lalonde and Marcus 2019a), therefore all species in any one place often share the same DNA barcodes, consequently barcodes cannot be used to delimit species (Brévignon and Brévignon 2012; Borchers and Marcus 2014; Gemmell *et al.* 2014). Although if species are identified morphologically beforehand it allows researchers to observe patterns of gene flow, hybridization, and biogeographic distributions of species (Moritz *et al.* 1987; Hebert *et al.* 2003b; Lalonde *et al.* 2018; Lalonde and Marcus 2019a; Lalonde and Marcus 2019b). This morphological identification followed by DNA barcoding technique is useful and I will use it to validate a restriction digest based method of genotyping (Chapter 2), expand biogeographic distribution patterns of a recent invasion event (Chapter 3), create biogeographic distributions in the Northern Hemisphere (Chapter 4), and investigate a ring species hypothesis (Chapter 4).

The use of mitochondrial DNA can provide insights into matrilineage-based work examining gene flow, dynamics of hybridization, population structure, population biology, biogeographic structure, and insights into taxa with brief evolutionary histories (Moritz *et al.* 1987; Hebert *et al.* 2003a; Kodandaramaiah and Wahlberg 2007; Lalonde *et al.* 2018; Lalonde and Marcus 2019a; Lalonde and Marcus 2019b). Already discussed above, are the limitations of using small fragment genes (such as the DNA barcoding fragment, ~658bp) in phylogenetic studies of recently diverged species. It has been suggested that using whole genome sequencing to obtain full mitochondrial genomes may aid in reducing ambiguities in species delimitation. In addition, delimitations may become clearer by comparing the pattern from mitochondrial DNA to patterns from rapidly evolving regions of the nuclear genome such as the internal transcribed spacer regions of the ribosomal repeat (Hebert *et al.* 2003a; Al-Nakeeb *et al.* 2017; Marcus 2018). Recent work using the whole mitochondrial genome has yielded some promising results in both vertebrate and invertebrate species (Krajewski *et al.* 2010; Williams *et al.* 2014; McCullagh 2016; Peters and Marcus 2017; Cong *et al.* 2020; Lalonde and Marcus 2020c) and some of the taxonomic ambiguities associated with the DNA barcode fragment have begun to subside. Illumina Next-generation sequencing of whole cell DNA extracts consistently yields for

Lepidopteran species a ~15.2 (kb) full mitochondrial genome sequence (McCullagh and Marcus 2015), and a complete 8-10 kb ribosomal RNA repeat (including the 18S, 5.8S, and 28S rRNAs and 2 ITS sequences) from the nuclear genome (Marcus 2018). These sequences, which include both slowly and rapidly evolving regions, allows for the production of robust phylogenetic trees that reflect the evolutionary history of the mitochondrion in recently diverged species such as *Junonia* (in which the mitochondrion is easily passed between species due to hybridization) (Borchers and Marcus 2014; Gemmell *et al.* 2014) as well as the nuclear genome (which is less likely to undergo introgression and replace the original sequence after hybridization). Creating phylogenetic reconstructions using both the full mitochondrial genome and rRNA repeat may aid in species delimitation, reconstructing evolutionary histories of species groups, and give insights into how biogeographic distributions of species have changed over time (Chapter 5).

***Genus Junonia***

The Buckeye butterflies were originally named based on the prominent eyespots that appear on their dorsal forewings, as well as the brown ground colour and cream-coloured subapical band that resembles the seeds of the buckeye tree (genus *Aesculus*), with the name in use since at least 1898 (Holland 1898, Munroe 1951). It must be noted that the genus *Junonia* were originally referred to as the 'peacock butterflies' by Holland (1898), but the association with this common name was later abandoned as it had already been associated with the European butterfly *Aglais io* and based on the common name associated with the North American *J. coenia* species the 'buckeye', this became the common name associated with all *Junonia* butterflies (Munroe 1951). The buckeye butterflies (genus *Junonia*) include ~48 described species (Table 1-1, (Kodandaramaiah and Wahlberg 2007; Gemmell and Marcus 2015b)) and are found on every continent except Europe and Antarctica, with the highest species diversity found in the tropics (Forbes 1928; Kodandaramaiah and Wahlberg 2007). *Junonia* species can be found in either forest (Old World species) (Williams 2021) or grassland/open habitats (Old World and all New World species) (Larsen 1999; Williams 2021). Many species prefer distinct host plants, and their distribution is based on larval host plant preferences (Brévignon 2004; Brévignon 2009; Brévignon and Brévignon 2012; Lalonde *et al.* 2018; Lalonde and Marcus 2019a).

This genus was found to have originated in Africa and is composed of both Old World and New World species (Kodandaramaiah and Wahlberg 2007; Kodandaramaiah 2009). The

taxonomic designations are rarely disputed in the Old World (29 species) but in the New World they have been difficult and have a complicated taxonomic history. This problem in species designations in the New World can be attributed to the lack of established and clear species descriptions (Schwartz 1989; Neild 2008; Calhoun 2010; Brévignon and Brévignon 2012; Gemmell *et al.* 2014). Many factors contribute to the lack of proper species identification including hybridization (Forbes 1928; Rutkowski 1971; Hafernik 1982; Minno and Emmel 1993; Lalonde and Marcus 2019a; Cong *et al.* 2020), geographic and seasonal phenotypic variation (Forbes 1928; Clark 1932; Mather 1967; Remington 1985; Smith 1991; Rountree and Nijhout 1995), close phenotypic resemblance of some morphs as well as cryptic species (Hafernik 1982; DeVries 1987; Glassberg 2007; Lalonde and Marcus 2019a), and vague species descriptions (Cramer 1775; Cramer 1780; Turner and Parnell 1985). For example, prior to 2008 only four separate *Junonia* species were thought to occur in the New World (Brévignon 2004; Neild 2008). Taxonomic work in the twenty-first century suggests that there are at least 18 New World *Junonia* species (Brévignon 2004; Brévignon 2009; Brévignon and Brévignon 2012; Gemmell and Marcus 2015; Lalonde and Marcus 2019a; Cong *et al.* 2020).

The genus *Junonia* is considered a valuable model in many fields of study not limited to, the evolution and development of wing colouration patterns (Kodandaramaiah 2009; Kodandaramaiah *et al.* 2013), herbivore-larval host plant interactions (Camara 1997; Knerl and Bowers 2013; Gemmell *et al.* 2014), and insect physiology and endocrinology (Otaki *et al.* 2005; Nijhout 2010; Martin and Reed 2014). However there has been little work done on the evolutionary history of the group, examining how these biological phenomena evolve. As *Junonia* is considered an important experimental model system, understanding the evolutionary history of the genus and having a universally understood taxonomic classification within the genus are important (Chapter 4 and Chapter 5). Unfortunately, many uncertainties in the taxonomic assignment of species still exist and these must be clarified before exploring hypotheses related to the evolutionary ecology of this genus. I will attempt to clarify the taxonomy in the New World, which has been long debated using both morphological and molecular-based methods using the species designations contained in Table 1-1.

**Table 1-1**. The 47 *Junonia* species and 14 subspecies considered in this thesis.

| Species |
|---|
| *J. adulatrix* Fruhstofer, 1903 |
| *J. agnesberenyiae* Sáfián, 2018 |
| *J. almana* (Linnaeus, 1758) |
| *J. ansorgi* Rothschild, 1899 |
| *J. artaxia* (Hewitson, 1864) |
| *J. atlites* (Linnaeus, 1763) |
| *J. coenia* Hübner, 1822 |
| *J. coenia bergi* Avinoff, 1926 |
| *J. coenia coenia* Hübner, 1822 |
| *J. chorimene* Guérin-Méneville, [1844] |
| *J. cymodoce* Cramer, [1777] |
| *J. cytora* Doubleday 1847 |
| *J. divaricata* C. & R. Felder, 1867 |
| *J. divaricata divaricata* C. & R. Felder, 1867 |
| *J. divaricata houlberti* Brévignon, 2008 |
| *J. divaricata mitraka* Brévignon, 2012 |
| *J. erigone* (Cramer, [1775]) |
| *J. evarete* Cramer, 1779 |
| *J. evarete dougueti* Brévignon, 2008 |
| *J. evarete evarete* Cramer, 1779 |
| *J. evarete occidentalis* C. & R. Felder, 1862 |
| *J. evarete pallens* C. & R. Felder, 1867 |
| *J. flirtea* (Fabricius, 1793) |
| *J. fuscencens* (Butler, 1901) |
| *J. genoveva* (Cramer, 1780) |
| *J. genoveva constricta* C. & R. Felder, 1867 |
| *J. genoveva genoveva* (Cramer, 1780) |
| *J. genoveva vivida* Forbes, [1929] |
| *J. goudoti* Boiduval, 1833 |
| *J. gregorii* Butler [1896] |
| *J. grisea* Austin & Emmel, 1998 |
| *J. hedonia* (Linnaeus, 1764) |
| *J. hierta* (Fabricius, 1798) |
| *J. hierta cebrene* (Stoneham, 1965) |
| *J. hierta hierta* (Fabricius, 1798) |
| *J. hilaris* C. & R. Felder, [1867] |
| *J. infuscata* C. & R. Felder, 1867 |
| *J. intermedia* (C. & R. Felder, 1862) |
| *J. iphita* (Cramer, [1779]) |
| *J. lemonias* (Linnaeus, 1758) |
| *J. litoralis* Brévignon, 2009 |
| *J. natalica* C. & W. Felder, 1862 |
| *J. neildi* Brévignon, 2004 |

*J. neildi neildi* Brévignon, 2004
*J. neildi varia*, Grishin 2020
*J. nigralis* Forbes [1929]
*J. nigrosuffusa* Barnes & McDunnough, 1916
*J. nigrosuffusa nigrosuffusa* Barnes & McDunnough, 1916
*J. nigrosuffusa stemosa* Grishin 2020
*J. oenone* Linnaeus, 1758
*J. orithya* (Linnaeus, 1758)
*J. orithya here* Lang, 1884
*J. orithya madagascariensis* Guenée, 1865
*J. orithya orithya* Linnaeus, 1758
*J. oscura* Neild, 2008
*J. pacoma* Grishin 2020
*J. rhadama* Boiduval, 1833
*J. sophia* (Fabricius, 1793)
*J. stygia* Aurivillius, 1894
*J. temora* Felder & Felder 1867
*J. terea* Crury, 1773
*J. timorensis* Wallace 1869
*J. touhilimasa* Vuillot, 1892
*J. vestina* C. & R. Felder, 1867
*J. vestina livia* Fruhstorfer, 1912
*J. vestina vestina* C. & R. Felder, 1867
*J. villida* (Fabricius, 1787)
*J. wahlbergi* Brévignon, 2008
*J. westermanni* Westwood 1870
*J. zonalis* C. & R. Felder, 1867
*J. zonalis michaelisi* Fruhstorfer, 1907
*J. zonalis swifti* Brévignon, 2004
*J. zonalis zonalis* C. & R. Felder, 1867

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## ***Life history traits of the genus Junonia***

All *Junonia* species are characterized morphologically based on their large conspicuous eyespots (Cramer 1775; Cramer 1780; Forbes 1928) which resemble markings found on the seeds produced by the buckeye tree (*Aesculus glabra*) from which they get their name (Holland 1898; Munroe 1951). Many species display phenotypic variation both seasonally and geographically, and in some cases closely resemble other forms making them morphological delimitation challenging (Forbes 1928; Clark 1932; Mather 1967; Remington 1985; DeVries 1987; Smith 1991; Rountree and Nijhout 1995; Glassberg 2007). The phenotypic variation that is displayed by *Junonia* happens in many other organisms, and is a form of phenotypic plasticity

that allows an organism to alter phenotypes based on environmental conditions using the same genotype (Brakefield 1996; Clarke 2017). The organisms that display such phenotypic plasticity are often called polyphenic species, and in butterflies this change is dependent on environmental factors during egg development (Brakefield and Zwaan 2011). In butterflies, this phenotypic change is usually induced based on photoperiod and temperature that is usually linked to seasonality (Brakefield 1984; Shapiro 1984; Brakefield and French 1993; Brakefield 1996; Simpson *et al.* 2011; Clarke 2017). Such changes in the phenotype that organisms display due to environmental conditions is termed seasonal polyphenism (Brakefield 1984; Brakefield 1996; Simpson *et al.* 2011; Clarke 2017). Seasonal polyphenism not only affects changes in phenotype but can also alter other aspects of an organisms biology such as metabolic rate, behaviour, physiology and life histories (Brakefield and Zwaan 2011; Clarke 2017). In butterflies these changes usually induce two differing forms depending on season, a wet season form that are usually active and brightly coloured with large eyespots, and a dry season form that usually have restricted mobility due to suboptimal environmental temperature and usually display cryptic colouration. Polyphenism sometimes led the same species to have been described as different taxa (Brakefield 1984; McLeod 1984; Shapiro 1984; Brakefield and French 1993). This phenotypic variation has made the delimitation of species a challenge in many groups including *Junonia*. Having comprehensive morphological and life history data on individual species can help clarify some of these problems (Lalonde *et al.* 2018; Lalonde and Marcus 2019a). In the case of the New World *Junonia*, where there is much variation, vague species descriptions, and a history of uncertainty in species identification, a comprehensive set of morphological and life history traits will aid in reducing the ambiguities that currently exist in specimen diagnosis (Chapter 4).

*Junonia* species are considered to be specialist feeders as their larvae feed on plants containing iridoid glycoside defensive compounds (Bowers 1984). Iridoid glycosides are prevalent in about 50 plant species that belong to the order Lamiales (El-Naggar and Beal 1980), and all *Junonia* larvae feed on species within this order (New World see Chapter 4 Electronic Supplement V(https://doi.org/10.34990/FK2/I6QUPN/FO5GVH), Old World (see Williams 2021)). Host plant species which contain iridoid glycoside compounds that *Junonia* species feed on belong to principle families within the Lamiales (Jensen *et al.* 1975; Bowers and Puttick 1986). The majority of plants that *Junonia* larvae feed on belong to the Scrophulariaceae

(figworts) and Plantaginaceae (plantain) families, while there are also 3 *Junonia* species that feed exclusively on *Avicennia germinans* (Black Mangrove, Acanthaceae) (Turner and Parnell 1985; Minno and Emmel 1993; Paulsen 1996; Glassberg 2007; Brévignon 2009; Brévignon and Brévignon 2012; Cong *et al.* 2020). Two specific iridoid glycoside compounds found within Lamiales, aucubin and catalpol, have been found to be feeding attractants and stimulants for *Junonia* larvae (Bowers 1984). *Junonia* sequester iridoid glycosides during the larval stages as a chemical defense against predators, but purge them during metamorphosis and adult *Junonia* lack these secondary compounds (Bowers and Stamp 1997). Additional work has shown that *Junonia coenia* sequesters more secondary compounds as larvae compared to many other butterfly species (Lampert and Bowers 2010), and generally adults of this species lay more eggs on plants with high concentrations of these compounds (Pereyra and Bowers 1988). These butterfly species have filled an ecological niche using plant species that most other herbivores generally avoid. Compiling a comprehensive list of host plant and habitat use in the New World where comprehensive species data are currently sparse (especially in South America) will help in identifying potential species habitats preferences, discern species distributions, and may aid in future specimen collecting (Chapter 4).

***Junonia mitochondrial haplotype groups***

The mitochondrial haplotype groups in the New World have been the focus of much molecular phylogenetic studies and have been well characterized in their differences when compared to Old World species. To date no distinct Old World haplotype groups have been assigned specific names, as has been done in the New World, but each of the species thus far has its own distinct mitogenome (Kodandaramaiah and Wahlberg 2007; Kodandaramaiah 2009; Pyrcz *et al.* 2021). There is only one Old World example of apparent mitogenome haplotype sharing that occurs between specimens from Asian and African populations of *J. hierta* and *J. orithya* based on geography rather than taxonomic species assignment (Kodandaramaiah and Wahlberg 2007). The New World buckeye butterfly mitochondrial haplotype groups are very well characterized and can be divided into three primary haplotype groups using DNA barcodes (two of which can be subdivided into subgroups) that are shared by many species (Kodandaramaiah and Wahlberg 2007; Kodandaramaiah 2009; Pfeiler *et al.* 2012; Borchers and Marcus 2014; Gemmell *et al.* 2014; Gemmell and Marcus 2015; Lalonde *et al.* 2018; Lalonde

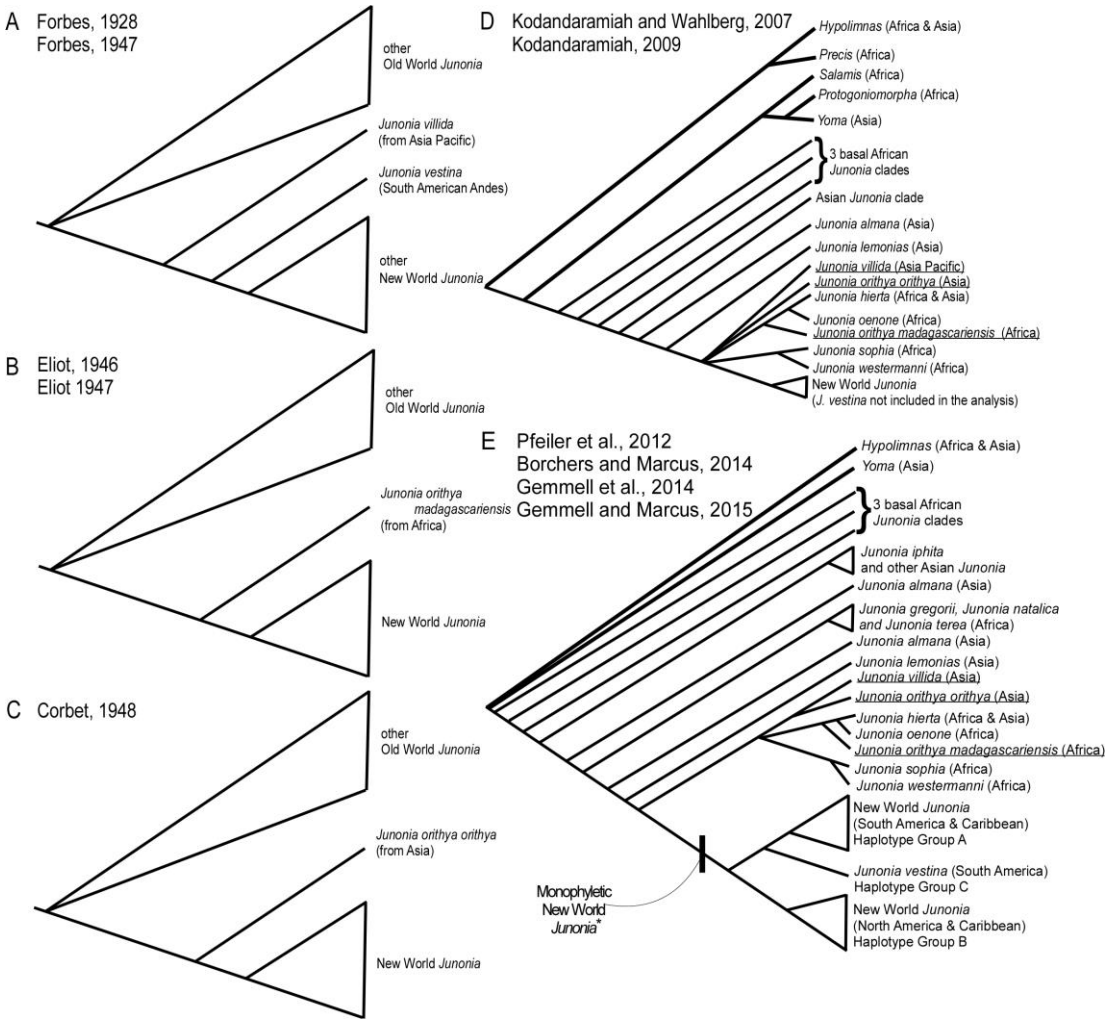
and Marcus 2019a; Cong *et al.* 2020). Haplotype group A, which is most commonly found in species from South America and the Caribbean, Haplotype group B which is most commonly found in species from North America, and Haplotype group C which is restricted to a high elevation Peruvian species, *Junonia vestina* (Pfeiler *et al.* 2012; Peters and Marcus 2017). Both Haplotype A and B can be subdivided into 2 distinct subgroups, with group A containing $A_1$ and $A_2$, and group B subdivided into B and $B^{CA}$. The distinct haplotype group $A_1$ has been found to only occur in *J. vestina* (a high elevation species), and all other species that carry haplotype group A, are considered to carry $A_2$ (Pfeiler *et al.* 2012). The distinct subgroup of haplotype group B haplotype associated with *Junonia* specimens from the American Southwest is known as haplotype $B^{CA}$, which was originally thought to only occur in a single cryptic taxon but based on previous work it is shared by all *Junonia* species found in the American Southwest and Mexico (Lalonde and Marcus 2019a). Haplotype group B is in most New World *Junonia* species and co-occurs with all other haplotype groups. The New World haplotype group C that is restricted to *J. vestina* is interesting as it is more closely related to the Old World *Junonia* clade containing *J. lemonias* from Asia than it is to any other haplotype group and does not even cluster with other New World Haplotypes (Peters and Marcus 2017). The distribution of haplotype groups A and B will be used to validate a restriction digest based method of mitochondrial haplotyping using this DNA barcode region (Chapter 2), expand our current knowledge of the invasion history of a *Junonia* in Florida (Chapter 3), attempt to examine specimens with ambiguous locality data from Chokoloskee, Florida (Chapter 3), and explore the biogeographic patterns of *Junonia* in the western hemisphere and test a ring species hypothesis proposed in South America (Chapter 4). The use of full mitochondrial genome data will be used to create the most comprehensive phylogeny of *Junonia* to date, consider the distribution of all 3 principle mitochondrial haplotype groups and their subgroups, and help to answer questions regarding the evolutionary history of the genus (Chapter 5).

***Brief taxonomic history for the genus Junonia***

Several morphology-based phylogenetic hypotheses have been proposed for the genus *Junonia* (Fig. 1-1 (A), (B), (C)), but have yet to be broadly accepted because they are mutually contradictory. These available morphology-based phylogenies also differ from the available molecular phylogenies based on a small number of mitochondrial (especially DNA barcode

sequences) or nuclear genes (Fig. 1-1 (D), (E)), and a few currently published mitogenome-based phylogenies (Peters and Marcus 2017; Cong *et al.* 2020; Lalonde and Marcus 2020c; Living Prairie Mitogenomics Consortium 2020). An additional contributing factor to a current lack in taxonomic consensus in *Junonia* is that in the New World, mitochondrial DNA haplotypes tend to be shared among all sympatric species in any given geographic location, resulting in identical mitochondrial gene sequences (Pfeiler *et al.* 2012; Gemmell *et al.* 2014; Gemmell and Marcus 2015; Lalonde *et al.* 2018; Lalonde and Marcus 2019a). There has also been a lack of consensus regarding the number of *Junonia* species although taxonomic assignments based on morphology have become less difficult in recent years due to work clarifying taxonomic designations for many species in the New World (Brévignon 2004; Brévignon 2009; Brévignon and Brévignon 2012; Gemmell *et al.* 2014; Lalonde *et al.* 2018; Lalonde and Marcus 2019a; Cong *et al.* 2020) but still remains a challenge due to the existence of undescribed species, some poorly described species, and potential cryptic species (Lalonde and Marcus 2019a; Cong *et al.* 2020). For molecular-based studies on *Junonia*, many have not considered the genus as a whole and present phylogenetic tree reconstructions containing either very few New World species (Fig. 1-? D, E, (Kodandaramaiah 2009; Clarke 2017; Pyrcz *et al.* 2021)) , or very few Old World species (Pfeiler *et al.* 2012; Gemmell *et al.* 2014; Gemmell and Marcus 2015; Peters and Marcus 2017; Cong *et al.* 2020; Lalonde and Marcus 2020c; Living Prairie Mitogenomics Consortium 2020). The most comprehensive molecular phylogeny to date is one that I generated during my PhD studies, which is a full mitochondrial genome sequence phylogeny consisting of 15 *Junonia* species (8 New World and 7 Old World species) (Lalonde and Marcus 2020c).

**Figure 1-1.** Previous phylogenies for the genus *Junonia*. A, B, and C represent the phylogenies based on morphological characteristics. D and E represent the molecular-based phylogenies (interpretation from McCullagh 2016).




The invasion events involving *Junonia* butterflies and specifically which lineages invaded the New World have been topics of interest for many decades. At least four separate taxa have been suggested as the sister taxon to the New World *Junonia*: *J. villida* from the Indo-Pacific (Forbes 1947), *J. orithya madagascariensis* from Africa (Eliot 1946; Eliot 1947), *J. orithya orithya* from Asia (Corbet 1948), and *J. lemonias* from Asia (McCullagh 2016; Peters and Marcus 2017). There has also been a debate if this invasion of the New World by *Junonia* was a single unique event (Forbes 1928; Eliot 1946; Eliot 1947; Forbes 1947; Kodandaramaiah and Wahlberg 2007; Neild 2008; Pfeiler *et al.* 2012) or was produced by multiple invasions involving a single or multiple Old World species (Gemmell and Marcus 2015; McCullagh 2016).

Based on recent studies using full mitochondrial genome sequence data, a trans-Pacific route is the most probable route for at least some of the invasion, and it is possible that the same lineage (*J. villida*) crossed the Pacific more than once to reach the New World (McCullagh 2016; Peters and Marcus 2017).

Based on previous studies, individual species of Old World *Junonia* were found to be monophyletic based on both morphological and molecular data, represented by bifurcation in phylogenetic trees suggesting that diversification in the Old World is an old event (Fig. 1-1) (Wahlberg *et al.* 2005; Kodandaramaiah and Wahlberg 2007; Kodandaramaiah 2009; Clarke 2017). Unlike Old World species, individual New World *Junonia* species fail to resolve into monophyletic clades based on most molecular studies to date (Fig. 1-1, (Pfeiler *et al.* 2012; Gemmell *et al.* 2014; Gemmell and Marcus 2015; McCullagh 2016; Peters and Marcus 2017; Lalonde *et al.* 2018; Lalonde and Marcus 2019a; Cong *et al.* 2020; Lalonde and Marcus 2020c)). This can be attributed to the fact that all New World *Junonia* species in any one geographic location share mitochondrial haplotypes, and that all populations of all species in that geographic location habitually showing the same proportion of each mitochondrial haplotype group (eg. 40% haplotype group A and 60% haplotype group B for each species in one place) (Gemmell and Marcus 2015; Lalonde and Marcus 2020a; Lalonde and Marcus 2020b). As discussed above, observations of many New World *Junonia* species have the capacity to interbreed, therefore hybridization and mitochondrial introgression events occur (Hafernik 1982; Paulsen 1996; Lalonde and Marcus 2019a; Cong *et al.* 2020). As a consequence, mitochondrial gene-based phylogenies are not useful for New World species delimitation (Kodandaramaiah and Wahlberg 2007; Kodandaramaiah 2009; Pfeiler *et al.* 2012; Gemmell *et al.* 2014; Gemmell and Marcus 2015; Peters and Marcus 2017; Lalonde *et al.* 2018; Lalonde and Marcus 2019a; Cong *et al.* 2020), although, these gene trees can still inform our understanding of *Junonia* evolutionary history if species determination is done using other characteristics. For example, the use of mitochondrial haplotype distributions can allow for the characterization of biogeographic patterns of genetic variation (Gemmell and Marcus 2015; Lalonde and Marcus 2019a; Lalonde and Marcus 2020a). This pattern of divergence that is observed in the New World *Junonia* indicates that enough time has passed to allow for sufficient genetic variation to have been achieved by these species, called reticulate evolution, which is characteristic of a rapid and recent species divergence (eg. (Marcus and McCune 1999; Cui *et al.* 2013; Keller *et al.* 2013)).

Additional sampling in the genus in both the Old and New Worlds using much larger fragments of DNA from both the mitogenome and nuclear rRNA repeat may allow for the resolution of the genus *Junonia* in the New World and give insights into the invasion history of the genus in the New World (Chapter 5).

***Thesis hypotheses and predictions:***

In this dissertation there are three main thesis questions that focus on the validation of a method of restriction digest based genotyping method (Chapter 2, 3), a ring species hypothesis (Chapter 4), and the creation of the most comprehensive molecular phylogeny for the genus to date (Chapter 5). All dissertation hypotheses, alternative hypotheses and associated predictions can also be found in Table 1-2 at the end of this Chapter.

The first question I investigated was the validation of a restriction digest based genotyping method and how it can be used reliably to answer questions related to an questions relating to the biogeographic history of a group. First mitochondrial DNA haplotyping by restriction digest was done to validate the usefulness of this technique for reliably recovering historical DNA from pinned museum specimens collected as early as 1813, and was used to determine if there was an age limit beyond which this technique is useful (Chapter 2). I hypothesize that restriction based haplotyping can be used reliably with museum specimens collected as early as 1813, and predict the successful recovery of mitochondrial haplotype groups will be achieved from all museum specimens in the dataset. This technique can greatly facilitate the exploitation of existing museum specimens to examine genetic variation over space and time. I then used additional museum specimens from a historical Florida collection to test whether they could be used to detect a possible invasion event (Chapter 3). Two questions were addressed in this chapter. The first is whether additional sampling from historical South Florida *Junonia* populations would recover additional localities for *J. zonalis* and evidence of Caribbean mitochondrial haplotypes that have not been found based on contemporary sampling. The second is whether these mitochondrial haplotypes be used to determine the correct locality data for historical insect specimens attributed to Chokoloskee, Florida (a locality with many anomalous species records). I predicted that mitochondrial genotyping of additional material from museum collections would allow the determination of geographic locality based on mitochondrial haplotype group frequencies.

The second research question I investigated was the hypothesis that the New World *Junonia* is a ring species using mitochondrial haplotyping by restriction enzyme digests. These results increased the knowledge of the diversity of species in Central and South America in this genus and documented interspecific gene flow in this genus (Chapter 4). I hypothesized that if the genus *Junonia* is a ring species in the New World, a disruption in genetic exchange would occur in Panama/Northern South America. I predicted that and abrupt change in the frequency in haplotype group A would occur with increasing latitude and haplotype group data (Fig. 1-2). If continuous genetic exchange is found a smooth transition in the frequency of haplotype group A would occur with increasing latitude (Fig. 1-3) falsifying the ring species hypothesis.

**Figure 1-2.** Representation of an abrupt shift in gene flow represented using the mitochondrial haplotype group A associated with latitude, that would add additional evidence that the genus *Junonia* is a ring species.

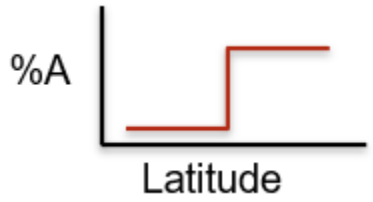



**Figure 1-3.** Representation of a continuous gradient in geneflow represented using the mitochondrial haplotype group A associated with latitude that would provide evidence that the genus *Junonia* is a not ring species.


The third research question focused on working to reconstruct the molecular phylogeny of the buckeye butterflies (Chapter 5). I focused on reconstructing the origins and evolutionary history of the recent and rapid diversification of New World buckeye butterflies (genus *Junonia*). The estimated 10-20 species that make up the New World section of this genus have evolved within the last 2.5 million years (McCullagh 2016). ). This work will reduce the ambiguities,

missing taxa, and polytomies that exist in current phylogenetic reconstructions of this genus based on small molecular data sets, also allowing for study of this explosive biological radiation will permit a broader understanding of the processes of speciation and adaptive radiation in general. There are three main research questions associated with this chapter. The first is that phylogenetic data sets composed of full mitochondrial genome and/or nuclear rRNA repeat sequences will fully resolve the New World *Junonia* butterflies. I predict that the genus *Junonia* will resolve into a monophyletic clade. The second is that the genus *Junonia* originated in Africa and the sister-taxon is either the genus *Protogoniomorpha* or *Precis*. The third question tests three hypotheses for the origin of the New World *Junonia*. The first is that the invasion of the New World occurred across the Atlantic by the lineage containing *J. orithya madagascarensis* and predicts that this lineage will resolve as the sister-taxon to the New World *Junonia* using mitogenome and rRNA repeat data. The second and third hypotheses are that the invasion of the New World occurred across the Pacific by either the lineage containing *J. orithya orithya* or the lineage containing *J. villida* lineage and predict that one of these lineages will resolve as the sister-taxon to the New World *Junonia* using mitogenome and rRNA repeat data.

**Table** 1-2. Hypotheses and the predictions to be investigated in this dissertation.

| **Geographic Variation in New World *Junonia* butterflies (Chapter 2, 3,4)** |
|---|
| $H_{1.1}$ Restriction digest based haplotyping can be used reliably with museum specimens collected as early as 1813 (Chapter 2). |
| Prediction: Successful recovery of mitochondrial DNA haplotype groups will be achieved using restriction-based methods from all museum specimens in the dataset including ones collected as early as 1813. |
| $H_{1.0}$ Restriction digest based haplotyping cannot be used reliably with museum specimens collected as early as 1813 (Chapter 2). |
| Prediction: Successful recovery of mitochondrial DNA haplotype groups will not be achieved for all sufficiently old museum specimens. |
| $H_{2.1}$ Additional Sampling from Florida populations will add additional insight into when *Junonia zonalis* and haplotype group A invasion into Florida (Chapter 3). |

Prediction: Additional group A haplotypes will be recovered with additional sampling of Florida *Junonia* museum specimens. Also, museum specimen will reveal additional localities for *J. zonalis* that are not known based on contemporary sampling.

$H_{2.0}$ Additional sampling from Florida populations will not add additional insight into the invasion history of *Junonia zonalis* and haplotype group A into Florida (Chapter 3).

Prediction: Additional group A haplotypes will not be recovered with additional sampling of Florida *Junonia* museum specimens. Also, museum specimen will not reveal additional localities for *J. zonalis*.

$H_{3.1}$ Analysis of additional material will allow for the determination if the historical material attributed to Chokoloskee, Florida a locality with many anomalous species records, are likely correct in their locality designations (Chapter 3).

Prediction: Additional Chokoloskee material from museum collections will allow for the determination that the specimens are correct in their locality designations based on mitochondrial haplotype group frequencies.

$H_{3.0}$ Analysis of additional material will not allow for the determination of whether historical material attributed to Chokoloskee, Florida are correct in their locality designations (Chapter 3).

Prediction: Additional Chokoloskee material from museum collections will not allow for the determination of where this material actually originated based on mitochondrial haplotype group frequencies.

$H_{4.1}$ The genus *Junonia* is a ring species in the New World with the disruption in genetic exchange occurring in Panama/Northern South America (Chapter 4)

Prediction: The genus *Junonia* will show an abrupt change in the frequency in haplotype group A with increasing latitude and haplotype group data will not show a smooth transition (Figure 1-2).

$H_{4.0}$ The genus Junonia is not a ring species in the New World and there is continuous genetic exchange in Panama/Northern South America. (Chapter 4)

Prediction: The genus *Junonia* will not show an abrupt change in the frequency of haplotype A frequency with increasing latitude and will show a smooth transition (Figure 1-3).

## Molecular Phylogeny of *Junonia* (Chapter 5)

$H_{5.1}$ Phylogenetic data sets composed of full mitochondrial genome and/or nuclear rRNA repeat sequences will fully resolve the New World *Junonia* butterflies.

Prediction: The genus *Junonia* will fully resolve into a monophyletic clade with phylogenetic reconstruction using full mitochondrial genome and rRNA repeat sequences.

$H_{5.0}$ Phylogenetic data sets composed of full mitochondrial genome sequences and/or nuclear rRNA repeat sequences will not resolve the New World *Junonia* butterflies.

Prediction: The genus *Junonia* will not resolve into a monophyletic clade and many polytomies of species with be apparent with phylogenetic reconstruction using full mitochondrial genome and rRNA repeat sequences.

$H_{6.1}$ The genus *Junonia* originated in Africa and the sister-taxon to *Junonia* is the genus *Protogoniomorpha*.

Prediction: Using mitogenome and rRNA repeat data the genus *Protogoniomorpha* will resolve as sister-genus to *Junonia*.

$H_{6.2}$ The genus *Junonia* originated in Africa and the sister-taxon to *Junonia* is the genus *Precis*.

Prediction: Using mitogenome and rRNA repeat data the genus *Precis* will resolve as sister-genus to *Junonia*.

$H_{6.0}$ The genus *Junonia* originated in Africa and a taxon other than *Protogoniomorpha* or *Precis* is sister to *Junonia*.

Prediction: Using mitogenome and rRNA repeat data the sister-genus to *Junonia* will be resolved, but the sister clade will be something other than *Protogoniomorpha* or *Precis.*

$H_{7.1}$ Invasion of the New World by *Junonia* occurred across the Atlantic by the *Junonia orithya madagascarensis* lineage.

Prediction: Using mitogenome and rRNA repeat data the genus *Junonia orithya madagascarensis* will resolve as sister-taxon to the New World *Junonia*.

$H_{7.2}$ Invasion of the New World by *Junonia* occurred across the Pacific by the *Junonia orithya* orithya lineage.

Prediction: Using mitogenome and rRNA repeat data the genus *Junonia orithya orithya* will resolve as sister-taxon to the New World *Junonia*.

$H_{7.3}$ Invasion of the New World by *Junonia* occurred across the Pacific by the *Junonia*

| | |
|---|---|
| *villida* lineage. | |
| | Prediction: Using mitogenome and rRNA repeat data the genus *Junonia villida* will resolve as sister-taxon to the New World *Junonia*. |
| $H_{7.0}$ Invasion of the New world by *Junonia* occurred across the Pacific by some other *Junonia* lineage. | |
| | Prediction: Using mitogenome and rRNA repeat data some other *Junonia* genus (e.g. *Junonia villida*) will resolve as sister-genus to the New World *Junonia*. |

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# Chapter 2: How old can we go? Evaluating the age limit for effective DNA recovery from historical insect specimens

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ML designed the study, did data collection, performed experiments, analyzed data and drafted the manuscript.

JM assisted with study design, provided laboratory space, research materials, and revisions to the manuscript.

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**Abstract**

Historical museum specimens are valuable for exploring population genetics and evolutionary questions because they can provide snapshots of morphological and genetic characteristics from populations over space and time. Unfortunately, DNA found in older museum specimens is frequently degraded, so obtaining genotypes from many individual samples necessary for rigorous molecular population genetic studies is challenging. Prior studies have varied greatly in their success at obtaining genotypes from older preserved insect material. Many well-intentioned collection curators have used research results showing poor preservation of DNA preserved in museum specimens to inform curatorial best practices, in some cases choosing not to allow DNA extraction by destructive sampling because in their estimation, the likelihood of success would be low. Recent methodological advances in DNA extraction, amplification, and genotyping have allowed some researchers to include mid-19$^{th}$ century samples in molecular genetic analyses. Here we present a robust, high-throughput, and low-cost DNA extraction and genotyping protocol for historical insect specimens employing restriction digests of PCR products followed by high sensitivity electrophoresis. Using this technique, we obtained mitochondrial haplotypes for 100% of 48 New World *Junonia* butterfly specimens (Nymphalidae) ranging in age from pre-1813 to 1909 and show that the haplotype frequencies obtained are statistically indistinguishable from 20$^{th}$ century and contemporary reference populations of *Junonia* (1,632 specimens) matched by geographic region. Since most extant insect specimens were collected after 1813, based on our findings we would expect that many or even most pinned specimens preserved in museum collections contain usable DNA for mitochondrial haplotyping.

## Introduction

Tracking the evolution of organisms over time can be challenging, as many evolutionary processes occur over time scales longer than the lifetimes of individual scientists. To eliminate this problem, some researchers have compared organisms from contemporary populations with organisms from historical populations using specimens found in museum collections (Goldstein and DeSalle 2003; Harper *et al.* 2006; Habel *et al.* 2009) . Museum collections are an invaluable resource because they contain specimens collected over many decades or centuries and represent an underutilized resource capable of providing snapshots of morphological (Carroll *et al.* 2005; Miller-Struttmann *et al.* 2015) and genetic characteristics (Goldstein and DeSalle 2003; Strange *et al.* 2009; Keyghobadi *et al.* 2013; Heintzman *et al.* 2014) from populations over time.

Historical museum specimens have been used to explore various population genetics and evolutionary questions in both vertebrates (Palkovacs *et al.* 2004; Austin and Melville 2006; Schmitt *et al.* 2018) and insects (Goldstein and DeSalle 2003; Harper *et al.* 2006; Habel *et al.* 2009; Saarinen and Daniels 2012; Keyghobadi *et al.* 2013; Heintzman *et al.* 2014). Vertebrate specimens are often large in size when compared to invertebrates, therefore tissues are relatively abundant so getting sufficient tissue for analysis has generally not been an issue, but the quality of the DNA can be dependent on preservation technique (Bouzat *et al.* 1998; Iudica *et al.* 2001). For many insects, specimens are small and little tissue is available for analysis; a partial leg or other small body part is often the only tissue available for destructive sampling and genetic analysis as the preservation of the remainder of the specimen may be required for identification by morphological characteristics (Watts *et al.* 2007). DNA quality recovered from preserved insect specimens is also dependent on preservation method as well as storage conditions (Dillon *et al.* 1996; Watts *et al.* 2007) and the amount of time specimens have been stored (Watts *et al.* 2007; Heintzman *et al.* 2014). Unfortunately, DNA found in older museum specimens is frequently degraded, so obtaining large numbers of genotypes from many individual samples necessary for rigorous molecular population genetic studies has often been unfeasible. Consequently, to date much of the work using limited sampling of historical insect museum specimens has focused on comparing levels of genetic variation in historical populations over time and with extant populations (Goldstein and DeSalle 2003; Harper *et al.* 2006; Habel *et al.* 2009).

The most successful studies based on museum specimens typically use either nuclear microsatellites (Strange *et al.* 2009; Saarinen and Daniels 2012), which are short amplified repetitive regions with alleles that vary in size and can be separated by electrophoresis, or fragments of mitochondrial DNA (mtDNA) (Goldstein and DeSalle 2003; Heintzman *et al.* 2014), which occurs at high copy number and can sometimes be sequenced from older museum specimens. The reliability of both of these techniques decreases with increasing age of the museum specimens making genotyping progressively difficult (Goldstein and DeSalle 2003; Mandrioli *et al.* 2006; Watts *et al.* 2007; Strange *et al.* 2009; Keyghobadi *et al.* 2013; Hernandez-Triana *et al.* 2014). To date the oldest insect specimens yielding microsatellite genotypes were collected in 1890's (Hymenoptera, Lepidoptera) (Harper *et al.* 2006; Habel *et al.* 2009; Strange *et al.* 2009). The earliest mtDNA sequences recovered by PCR and Sanger sequencing are from Coleoptera collected in the early 1870's (Goldstein and DeSalle 2003; Heintzman *et al.* 2014), and Next-generation sequencing techniques have recently been used to recover mtDNA sequences from Coleoptera collected in 1859 (Sproul and Maddison 2017) and from Lepidoptera collected as early as 1864 (e.g. *Ctimene basistriga* (Geometridae), (Prosser *et al.* 2016)), but mtDNA sequence recovery in other insect orders has only been successful to the 1940's (Watts *et al.* 2007; Keyghobadi *et al.* 2013; Hernandez-Triana *et al.* 2014). Other Next-generation sequencing-based techniques such as RAD-tagging have been successful for genotyping insect specimens collected as long ago as 1910 (Hymenoptera, (Tin *et al.* 2014)). Evaluating success rates for genotyping from museum specimens is often ambiguous as some studies report only successful data collection and not the number of specimens where genotyping was unsuccessful (Harper *et al.* 2006; Habel *et al.* 2009; Saarinen and Daniels 2012). The question remains, how old can we go before DNA degradation in specimens has progressed to the point where recovering genotypes becomes unlikely or impossible?

By addressing this question, we can help collection curators develop best practices for selecting which material is suitable for destructive sampling for genotyping, and which material is too old for such purposes and is best preserved intact. Here we focus on mitochondrial genotyping, since it is widely used for species identification (Hebert *et al.* 2004; Janzen *et al.* 2005; Meusnier *et al.* 2008; Pfeiler *et al.* 2012; Bartomeus *et al.* 2018), and because samples that fail to yield mitochondrial genotypes typically also fail to yield nuclear genotypes (Gemmell *et al.* 2014). We have developed a new method that combines advantages of the high copy number

of mtDNA with the robustness of fragment-based methods used for genotyping microsatellites (Gemmell and Marcus 2015; Lalonde *et al.* 2018; Lalonde and Marcus 2019a; Lalonde and Marcus 2019b). Using an effective DNA extraction protocol, PCR, a restriction fragment-based genotyping assay, and a very sensitive capillary electrophoresis instrument (Qiagen QiAxcel) for detecting DNA fragments, we can go from tissue sample to mitochondrial genotype in as little as one day at ~1/10th the cost of fluorescent dye-terminated Sanger DNA sequencing ($0.83 USD versus $9.12 USD per sample at July 2019 prices and currency exchange rates). It should be noted that this new genotyping method does not involve sequencing DNA, but instead assigns mitochondrial genotypes based on the presence or absence of sequence- and haplotype-specific restriction enzyme cut sites in the DNA fragments being evaluated (which were already known for each haplotype based on prior exploratory work that included conventional DNA sequencing (Borchers and Marcus 2014; Gemmell *et al.* 2014; Peters and Marcus 2017; Lalonde *et al.* 2018)). In our previous work, the fragment-based genotyping technique has worked with 100% efficiency for over 1,800 specimens collected in the 20$^{th}$ century and contemporary specimens (1910-2016) from the butterfly genus *Junonia* (Lepidoptera: Nymphalidae, Table 2-1), as well as for a handful of 19$^{th}$ century specimens, with the oldest genotyped museum specimen dating from 1866 (Lalonde and Marcus 2019a; Lalonde and Marcus 2019b).

**Table 2-1**. New World *Junonia* species sampled in historical and reference populations.

| *Species* |
|---|
| *J. coenia* Hübner, 1822 |
| *J. divaricata* C. & R. Felder, 1867 |
| *J. evarete* Cramer, 1779 |
| *J. evarete dougueti* Brévignon, 2008 |
| *J. evarete evarete* Cramer, 1779 |
| *J. evarete occidentalis* C. & R. Felder, 1862 |
| *J. evarete pallens* C. & R. Felder, 1867 |
| *J. flirtea* (Fabricius, 1793) |
| *J. fuscencens* (Butler, 1901) |
| *J. genoveva* (Cramer, 1780) |
| *J. genoveva constricta* C. & R. Felder, 1867 |
| *J. genoveva genoveva* (Cramer, 1780) |
| *J. genoveva vivida* Forbes, 1928 |

*J. grisea* Austin & Emmel, 1998
*J. hilaris* C. & R. Felder, 1867
*J. infuscata* C. & R. Felder, 1867
*J. litoralis* Brévignon, 2009
*J. neildi* Brévignon, 2004
*J. nigrosuffusa* Barnes & McDunnough, 1916
*J. oscura* Neild, 2008
*J. wahlbergi* Brévignon, 2008
*J. zonalis* C. & R. Felder, 1867

New World species not sampled for this study
*J. vestina* C. & R. Felder, 1867

In the New World *Junonia*, due to retained ancestral polymorphism and/or lateral transfer between species, mitochondrial haplotypes are not species-specific (Borchers and Marcus 2014; Gemmell *et al.* 2014; Gemmell and Marcus 2015; Lalonde *et al.* 2018). However, regardless of species, all of the *Junonia* from any one locality typically share mitochondrial haplotype frequencies for the two most common haplotype groups (A and B) found in most of the Western hemisphere (Pfeiler *et al.* 2012; Gemmell and Marcus 2015; Peters and Marcus 2017; Lalonde *et al.* 2018; Lalonde and Marcus 2019b). It is noteworthy that haplotype group A is virtually absent from all species of North American *Junonia* except in south Florida (Gemmell and Marcus 2015; Lalonde *et al.* 2018; Lalonde and Marcus 2019a; Lalonde and Marcus 2019b). Two additional rarer *Junonia* haplotypes are geographically restricted, with haplotype C known only from the Peruvian Andes, and a distinct variant of haplotype group B, haplotype $B^{CA}$ is found commonly in the American Southwest and Mexico (Gemmell and Marcus 2015; Lalonde and Marcus 2019b). In most localities these haplotype group frequencies are stable over many decades, except for when a recent invasion event has occurred (Lalonde and Marcus 2019a). These mitochondrial haplotype groups are easily distinguished in our restriction digest assay and permitting comparison between allele frequencies from historical and recent *Junonia* populations from the same geographic locations. If this very robust assay fails to recover genotypes from high copy number mitochondrial DNA, other genotyping methods (including Next-generation sequencing methods that require long amplicons) that are more sensitive to poor DNA preservation and/or low DNA concentration probably will also fail in for those specimens. Here we extensively sample the earliest New World *Junonia* specimens available from museum collections to determine if there is an age beyond which one is unable to consistently assign

mitochondrial genotypes in insect specimens in the hope that it will help to inform future destructive sampling guidelines used by researchers and museum curators.

## Materials & Methods

**Specimen Collection and Preparation**. To evaluate the maximum insect specimen age that still permits mitochondrial genotyping, we selected 48 New World *Junonia* butterfly specimens collected prior to 1910 (ranging in age from pre-1813 to 1909, Electronic supplement I, https://doi.org/10.34990/FK2/I6QUPN/027KJ0) to compare with specimens from reference populations that we have genotyped previously for other projects (Electronic supplement II, https://doi.org/10.34990/FK2/I6QUPN/GZ95OE, (Lalonde *et al.* 2018; Lalonde and Marcus 2019a; Lalonde and Marcus 2019b)). To reduce possible biases due to differences in storage techniques, we sampled specimens from five natural history museum collections (American Museum of Natural History (AMNH), Illinois Natural History Survey (INHS), Natural History Museum London (NHMUK), Smithsonian National Museum of Natural History (USNM), Yale Peabody Museum of Natural History (PMNH)) and two private collections (Thomas W. Turner and specimens maintained as part of the research collection within the Marcus laboratory) for inclusion in our data set.

A single mesothoracic (middle) leg was removed from each specimen unless it was missing from the specimen in which case a metathoracic (hind) leg was removed instead. Each leg sample was bisected halfway along the proximal-distal axis and one half of the leg was used for DNA extraction while the other was placed in a labeled envelope and frozen at -20˚C as a tissue voucher. DNA extraction was performed manually as previously described (Gemmell and Marcus 2015; Lalonde and Marcus 2019a) in an isolated clean laboratory where no other molecular work was taking place with the Qiagen DNEasy Blood and Tissue kit (Qiagen, Düsseldorf, Germany) according to the manufacturer's instructions with the modifications as described previously and summarized below (Lalonde and Marcus 2019a). Leg tissue was ground with a ceramic mortar and pestle in 180 µl of "mouse tail-tip" lysis buffer (1% SDS, 0.1M NaCl, 0.1M EDTA, 0.05M Tris and deionized distilled water) instead of Qiagen tissue lysis buffer ATL to increase the recovery of extracted DNA. Once homogenized, 20 µl of proteinase K (Qiagen, 600 mU/mL) was added and the mixture was incubated for one hour in a

55ºC water bath until tissue was lysed. Negative (no tissue) DNA extraction controls were also prepared using the same reagents and techniques. Final DNA elutions were recovered in two separate 100 μL aliquots. Only first elution aliquots were used for measuring DNA concentration and for genotyping.

Sample DNA concentrations in our laboratory are routinely evaluated using a Nanodrop 2000 spectrophotometer (Nanodrop, Wilmington, Delaware, USA), but these measurements can be unreliable at low DNA concentrations as might be expected from extracts derived from older museum specimens. For this reason, the DNA concentrations from the 48 historical DNA samples were also evaluated in triplicate using the Invitrogen Quant-iT 1X dsDNA HS (High-Sensitivity) Assay Kit and a Life Technologies Qubit 3.0 fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to the manufacturer instructions. Then, DNA extracts were subsequently stored at -20˚C until use.

**Genotyping Assay.** Fragments of cytochrome c oxidase subunit I (*COI*) were amplified by polymerase chain reaction (PCR) from 1 or 2 μL of the first elution DNA extract from each sample. All samples were first amplified using the primers LCO1490/HCO2198 (Fig. 2-1, Table 2-2, Folmer *et al.* 1994). PCR reaction conditions used were as follows: 95˚C for 5 minutes; 35 cycles of 94˚C for 1 minute, 46˚C for 1 minute, 74˚C for 1 minute, 94˚C for 1 minute; and a final extension for 5 minutes at 72˚C, then a 4˚C hold. To visualize obtained amplification products from samples, they were run on a QIAxcel Advanced capillary electrophoresis instrument (Qiagen) as reported previously (Gemmell and Marcus 2015). Negative control amplifications that used distilled deionized water (instead of extracted DNA) were included within each PCR reaction. Whenever amplification was detected in the negative controls, which suggested contamination, all PCR reactions from the experiment were discarded and subsequent PCR amplification was repeated with fresh reagents. The protocols for PCR, verification of PCR products, and restriction digests were used for all experiments described here, unless otherwise specified. Because the DNA in older museum material is degraded, amplification with LCO1490/ HCO2198 primers, which produce a 709 bp amplicon, was unsuccessful for 47 of 48 historical samples, so they were reamplified using primer pairs that produced smaller amplification products: miniCOIF2/ HCO2198 (569 bp amplicon, Gemmell *et al.* 2014),

miniCOIF2/ miniCOIR3 (501 bp amplicon, Gemmell and Marcus 2015), or miniCOIF2/ miniCOIR2 (339 bp amplicon, Gemmell *et al.* 2014) and miniCOIF3/miniCOIR3 (258 bp amplicon, Gemmell and Marcus 2015) (Fig.2-1, Table 2-2).

**Figure 2-1.** Restriction digest map for PCR products generated using *cytochrome oxidase subunit I* (*COI*) primers to determine mitochondrial haplotype group. The cut sites for BamHI, AflIII, and BseYI are shown using a vertical bar. Haplotype group A alleles have one BamHI cut site while, group B have one AflIII and one BseYI cut site. The associated haplotype group is displayed above the vertical bar and the position of the cut site is indicated below.

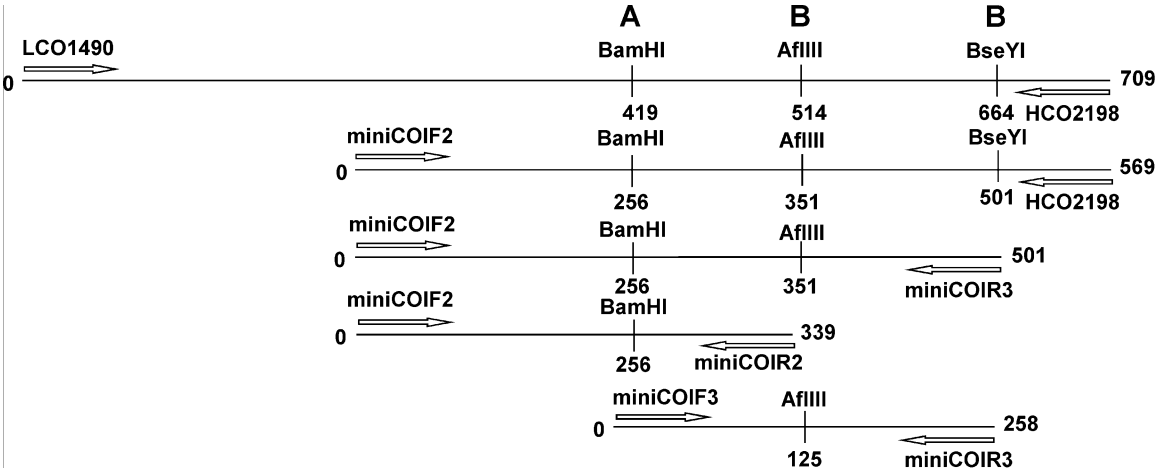
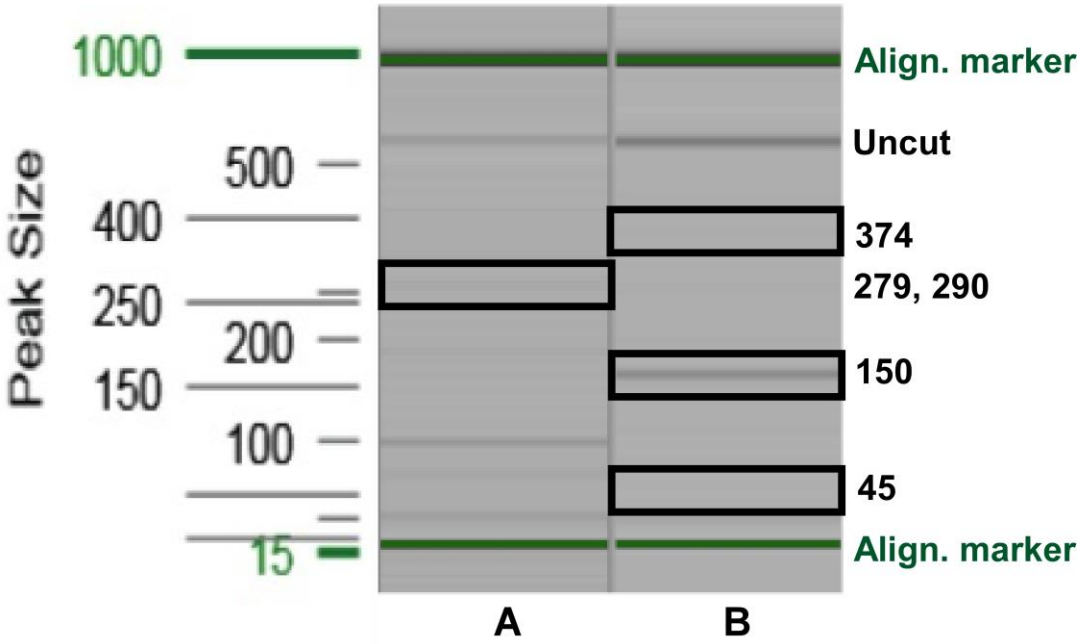



**Table 2-2.** Primer sequences used for PCR in amplification and reamplification processes to obtain cytochrome c oxidase subunit I (*COI*) fragments for haplotype determination. The forward primer is designated with (F) while the reverse primer is designated with (R). All sequences are given from 5' to 3'.

| Primer | Sequence | Source |
|---|---|---|
| LCO1490 (F) | GGT CAA ATC ATA AAG ATA TTG G | (Folmer *et al.* 1994) |
| HCO2198 (R) | TAA ACT TCA GGG TGA CCA AAA AAT CA | (Folmer *et al.* 1994) |
| miniCOIF2 (F) | ATA CTA TTG TTA CAG CCT CAT GC | (Gemmell *et al.* 2014) |
| miniCOIR2 (R) | TGT TGT AAT AAA ATT AAT AGC TCC | (Gemmell *et al.* 2014) |
| miniCOIF3 (F) | CCC CAC TTT CAT CTA ATA TTG C | (Gemmell *et al.* 2014) |
| miniCOIR3 (R) | TAT TTC GAT CTG TTA AAA GTA TAG | (Gemmell and Marcus 2015) |

Once PCR products were obtained, diagnostic restriction enzyme digests were performed using AflIII, BamHI, and BseYI restriction endonucleases (New England Biolabs (NEB), Ipswich, MA, USA) to reliably identify the haplotype group of each specimen as previously described (Gemmell and Marcus 2015). The digested products were resolved using a QIAxcel Advanced instrument as described above for evaluating PCR products. Haplotypes were assigned based on the size of the DNA fragments obtained. Haplotype Group A specimens have a single BamHI cut site, which produces 2 bands in this digest while amplifications of Haplotype Group B specimens lack this BamHI site, but are cut once each with AflIII, and BseYI and produce 3 distinct bands differing in size from the BamHI cut site (Fig. 2-2). When the smallest PCR products (miniCOIF2/ miniCOIR2 and miniCOIF3/miniCOIR3) were used for genotyping, they each contain only one cut site, so both were amplified, and each product was digested separately in order to determine the mitochondrial haplotype. These assays were supplemented by digests with additional restriction enzymes to separate haplotype group B from the related haplotype subgroup $B^{CA}$ for specimens from geographic regions (Mexico, USA: California) where this subgroup occurs (Lalonde and Marcus 2019a). Haplotype C has not been documented from any of the New World regions where historical *Junonia* specimens have been sampled, but PCR products from haplotype C genotypes would fail to cut in these assays.

**Figure 2-2.** Sample output from Qiagen QiAxcel Advanced capillary electrophoresis instrument showing the expected fragments produced by restriction digests of LCO1490/HCO2198 amplification products from *Junonia* butterflies carrying mitochondrial haplotype groups A and B. The QiAxcel instrument does not consistently resolve the 279 bp and 290 bp bands under the settings used, so they often co-migrate and appear as a single band.




Haplotype group B frequencies of historical *Junonia* specimens (n=48) were compared with haplotype frequencies of 20th century and contemporary specimens from reference populations (n=1,632) matched by collection locality. Standard error for each proportion was calculated using standard methods by taking the square root of ((proportion A*(1-proportion A))/total sample size) for each locality (Stuart 1963). Haplotype frequencies for paired historical and reference collections were evaluated by a paired t-test (Snedecor and Cochran 1989). The power analysis for this t-test was calculated using Statulator statistical software (Dhand and Khatkar 2014).

**DNA Sequencing and Phylogenetic Analysis.** To verify that our genotyping assay was producing valid genotypes, we sequenced PCR amplification products from the two oldest samples in our data set, two pre-1813 specimens of *J. genoveva* collected in Suriname and

preserved in the Cornelis Van Lennep (1751-1813) collection, now in the collections of the Natural History Museum London. These specimens may have been collected several decades before 1813 (Neild 2008). PCR fragments were sequenced in both directions using Sanger dideoxy sequencing and ABI BigDye V3.1 Dye Termination sequencing chemistry (Applied Biosystems, Carlsbad, California, USA) as previously described (Borchers and Marcus 2014). Sequencing reactions were analyzed on an ABI 3730*xl* automated DNA analyzer.

Sequence reads were aligned, checked for quality, and edited (primer sequences removed) using Sequencher 5.1 software (GeneCodes Corporation, Ann Arbor, Michigan, USA). Edited sequences were converted to FASTA format and aligned in Clustal Omega (Sievers *et al.* 2011) alongside 10 representative *COI* sequences from the A and B haplotype groups, plus a sequence from *J. villida* from Australia as an outgroup, from prior phylogenetic studies (Borchers and Marcus 2014; Gemmell *et al.* 2014; Gemmell and Marcus 2015; Peters and Marcus 2017). Sequence alignments were converted to NEXUS format for phylogenetic analysis.

Appropriate maximum likelihood models were selected for each phylogenetic analysis using jModelTest2.1.7 (Darriba *et al.* 2012) and likelihood ratio tests (Huelsenbeck and Rannala 1997). A GTR+I+G model (Rodríguez *et al.* 1990) (I = 0.702 , G = 0.549) was determined to be the best fit for the dataset. Sequences were analyzed using PAUP* 4.0b8/4.0d78 (Swofford 2002) with default settings unless otherwise specified. The data set was analyzed using both parsimony and maximum likelihood optimization criteria. In each case, 1 million replicate heuristic searches were conducted with random taxon addition order, tree bisection and reconnection (TBR), and all of the best trees retained. For each dataset, 1 million bootstrap replicates were also performed using both maximum likelihood (using the GTR+I+G model described above) and parsimony algorithms with fast step-wise additions and retention of groups consistent with 50% majority rule consensus.

## Results

The fluorometric measurements of DNA concentrations from the 48 historical (pre-1910) *Junonia* butterfly specimen extracts ranged from 0.060 ng/μL to 0.910 ng/μL (Electronic

Supplement I, https://doi.org/10.34990/FK2/I6QUPN/027KJ0, mean 0.307 ng/μL, SD 0.196 ng/μL) for a first elution aliquot total recovery of between 6 and 96 ng DNA. Haplotype data was obtained from all 48 specimens (100% genotyping success rate for specimens dating from pre-1813 to 1909) from 11 geographic regions in the New World (Table 2-3) using an efficient DNA extraction protocol, PCR, diagnostic restriction digests, and high sensitivity capillary electrophoresis-based genotyping. The PCR primers that produced amplicons and were used to genotype each specimen are listed in Table S1. Overall, 1 sample (collected in 1879 in California) produced a 709 bp amplicon, 17 samples produced a 569 bp amplicon, 28 samples produced a 501 bp amplicon, and 2 samples (the two oldest specimens in our study, collected pre-1813 in Suriname) were genotyped from 339 bp and 258 bp amplicons. No tissue DNA extraction controls or negative PCR controls produced amplicons for any of the primer pairs. For comparison with the experimental results from historical specimens, we gathered haplotype group frequency information from contemporary (20$^{th}$ and 21$^{st}$ century) *Junonia* reference populations from each of these 11 regions (total 1,632 samples, Electronic Supplement 2, https://doi.org/10.34990/FK2/I6QUPN/GZ95OE). The proportion of haplotype group B was used as a metric to compare historical and reference populations.

**Table 2-3.** Haplotype group B frequencies of historical *Junonia* specimens (n=48) compared with haplotype frequencies of 20th century and contemporary specimens from reference populations (n=1,632) matched by collection locality. When the number of historical specimens in a sample is greater than 1, the sample size is indicated in parentheses.

| Species | Locality | Date | Sample Size | Proportion B | 1SE | Reference Population | Sample Size | Proportion B | 1SE |
|---|---|---|---|---|---|---|---|---|---|
| *J. genoveva* | **Brazil: Amazonas, Rio Calary-Uaupes** | **1906** | **5** | **0.000** | **0.000** | **NW Brazil** | **45** | **0.378** | **0.072** |
| *J. coenia* (3), *J. nigrosuffusa* X *coenia* | USA: MO, St. Louis | 1900 | 4 | 1.000 | 0.000 | | | | |
| *J. coenia* | USA: DC | 1890 | 1 | 1.000 | 0.000 | | | | |
| | **Total USA: MO, DC** | **1890-1900** | **5** | **1.000** | **0.000** | **USA: MO, KY, NC** | **16** | **1.000** | **0.000** |
| *J. genoveva hilaris* | **Bolivia: Cochabamba, Yunca de Espiritu Santo** | **1888-1889** | **2** | **0.000** | **0.000** | **Bolivia** | **15** | **0.000** | **0.000** |
| *J. nigrosuffusa* X *coenia* | Mexico: Puebla | 1888 | 1 | 0.000 | 0.000 | | | | |
| *J. coenia* | Mexico: Orizaba | 1887 | 1 | 1.000 | 0.000 | | | | |
| | **Total Mexico**[a] | **1887-1888** | **2** | **0.500** | **0.354** | **Mexico** | **136** | **0.765** | **0.036** |
| *J. zonalis* | Trinidad and Tobago: Trinidad, Carenage | 1909 | 2 | 0.000 | 0.000 | | | | |
| *J. zonalis* | Trinidad and Tobago: Trinidad, | 1886 | 2 | 0.000 | 0.000 | | | | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Broadway | | | | | | | | |
| | **Total Trinidad** | **1886-1909** | **4** | **0.000** | **0.000** | **Trinidad** | **16** | **0.688** | **0.116** |
| *J. evarete fuscencens* (2), *J. evarete basifusca* | **Ecuador: environs de Loja** | **1885-1888** | **3** | **0.333** | **0.272** | **Ecuador** | **79** | **0.393** | **0.055** |
| *J. grisea* | **USA: CA, Los Angeles Co., Santa Monica**[a] | **1879** | **1** | **1.000** | **0.000** | **USA: CA** | **174** | **0.201** | **0.030** |
| *J. divaricata* (7), *J. genoveva* | Venezuela: Bolivar, Cuidad | 1895-1898 | 8 | 0.375 | 0.171 | | | | |
| *J. genoveva* | Venezuela: Rio Suezpure | 1891 | 1 | 0.000 | 0.000 | | | | |
| *J. evarete* (4), *J. wahlbergi* (2), *J. zonalis* (3) | Venezuela: San Estevan, pre Puerto Cabello | 1877 | 9 | 0.444 | 0.166 | | | | |
| | **Total Venezuela** | **1877-1898** | **18** | **0.389** | **0.115** | **Venezuela** | **77** | **0.512** | **0.057** |
| *J. coenia* | USA: Florida | 1894 | 1 | 1.000 | 0.000 | | | | |
| *J. coenia* | USA: Florida | 1875 | 1 | 1.000 | 0.000 | | | | |
| | **Total USA: FL** | **1875-1894** | **2** | **1.000** | **0.000** | **USA: FL** | **916** | **0.773** | **0.014** |
| *J. zonalis* | Cuba: Santiago de Cuba | 1905 | 1 | 1.000 | 0.000 | | | | |
| *J. zonalis*, *J. coenia* X *zonalis* | Cuba: Tanamo | ~1903[b] | 2 | 0.500 | 0.354 | | | | |
| *J. zonalis* | Cuba | ~1866[c] | 1 | 1.000 | 0.000 | | | | |
| | **Total Cuba** | **1866-1905** | **4** | **1.000** | **0.000** | **Cuba** | **155** | **0.620** | **0.039** |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *J. genoveva* | **Suriname** | **Pre-1813**[d] | **2** | **1.000** | **0.000** | **Suriname** | **3** | **0.330** | **0.271** |

[a]Historical specimens and population also screened for haplotype subgroup $B^{CA}$ as described in (Lalonde and Marcus 2019a).

[b]Specimen labels undated. Appear to be part of a group of Lepidoptera specimens collected in Tanamo, Cuba by William Schaus (1858-1942) in 1903 (Schaus 1904).

[c]Specimen label undated. Specimen was part of a group of 128 Cuban insect specimens donated to the Yale University

by E. Suffert of Havana, Cuba in 1866 in exchange for 150 insect specimens from New England (Hawley *et al.* 1867).

[d]Specimen labels undated. Collection date assigned as pre-1813 because these 2 specimens originate from the insect collection of Cornelis Van Lennep (1751-1813) (Neild 2008). These specimens may actually be several decades older than 1813 (Neild 2008)

Two sampled populations (Bolivia and USA: MO, DC) contain only one mitochondrial haplotype each, though it is a different haplotype in each case. For both of these populations, the same haplotype was recovered in all historical and reference samples. The remaining nine reference populations are polymorphic for mitochondrial haplotype. For three historical populations (Mexico, Ecuador, and Venezuela) we recovered both of the expected haplotype groups based on the corresponding reference population haplotype frequencies, and in each case the observed haplotype frequencies in historical and reference populations are within 1 standard error. In the remaining six historical populations, only one mitochondrial haplotype was recovered from each population; the most common reference population haplotype was recovered in three historical populations (Brazil, USA: FL, and Cuba,) and while a rarer haplotype from the reference populations was recovered in three historical populations (Brazil, Suriname, USA: CA). Haplotype $B^{CA}$ was only recovered from historical samples from the regions where it had been previously documented in contemporary samples (Mexico, California, and adjacent US states) and as expected based on the geographic origins of the historical samples (none were from Peru), haplotype C was not recovered from any historical specimens. Overall, a paired t-test (2 tails, 10 d.f.) between paired historical and reference populations from the same geographic regions showed no statistically significant difference between the two data sets ($t = 0.38$, $p = 0.71$) suggesting that differences in haplotype frequencies between historical and more contemporary sampling of these geographic regions is within the expected variation due to sampling error. The conventional threshold for statistical power is often set at 0.80 ($\beta \leq 0.2$) (Di Stefano 2003). The statistical power of this t-test was determined to be 0.89 (Dhand and Khatkar 2014), meeting this criterion and indicating that the probability of making a type II error and wrongly failing to reject the null hypothesis is $\beta = 0.11$ or 11%.

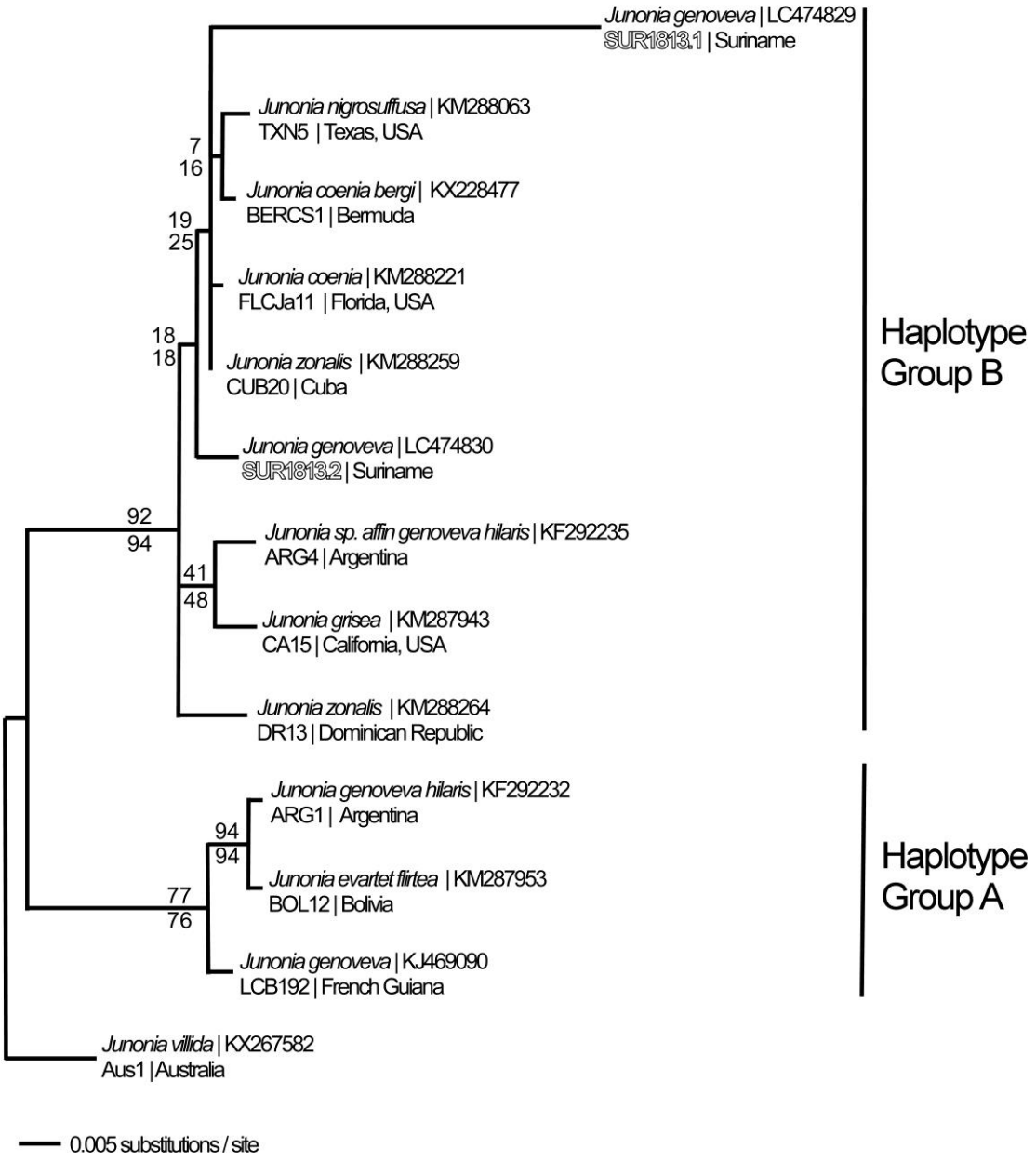
Finally, we verified the genotypes of the two oldest specimens in our data set by sequencing and phylogenetic analysis. We recovered Sanger DNA sequences from both pre-1813 *J. genoveva* specimens from Suriname (150 bp from SUR1813.1 and 404 bp from 1813.2) and deposited them in DDBJ (DNA Data Bank of Japan, accession numbers LC474829-LC474830). In addition to the short read-lengths which are consistent with fragmented DNA templates, the recovered sequences consistently showed low amplitude

primary peaks at each sequence position as well as prominent secondary peaks consistent with nucleotide deamination: cytosine undergoing deamination to uracil, producing apparent C to T transition mutations (Hassanin *et al.* 2009) and adenosine undergoing deamination to hypoxanthine, producing apparent A to G transition mutations (Zhang *et al.* 2013) in the resulting sequences. Also present, especially in the SUR1813.1 sequence, are apparent mutations that at sites that are otherwise invariant in New World *Junonia COI* sequences, all of which are consistent with previous examinations of historical insect samples in which sequence degradation was high (Kanda *et al.* 2015; Sproul and Maddison 2017).

Phylogenetic analysis of these sequences with examples of haplotype groups A and B show that both historical sequences are within the haplotype group B clade, as predicted by the restriction digest genotyping assay (Fig. 2-3). Heuristic parsimony searches produced 74 equally most parsimonious trees (score 63). The seven optimal trees found by maximum likelihood heuristic searches (score 1270.84395) were a subset of the parsimony trees. All of these optimal trees differed from one another only in the arrangement of sequences within haplotype group B. Parsimony and maximum likelihood bootstrap support for each of the haplotype groups was very strong, though the bootstrap support within haplotype group B was weak. The long branch-length associated with SUR1813.1 in the maximum likelihood tree depicted in Fig. 2-3 is likely a further reflection of apparent "mutations" associated with DNA degradation during the more than 200 years that this historical specimen was stored in museum collections.

**Figure 2-3.** One of 7 optimal maximum likelihood trees (GTR+I+G model, I= 0.702, G= 0.549, likelihood score 1270.84395) showing the monophyly of *Junonia* haplotype groups A and B, and the unambiguous assignment of *COI* sequences from *J. genoveva* collected in Suriname prior to 1813 (SUR1813.1 and SUR 1813.2) to haplotype group B. Parsimony analysis of the same data set recovered 74 equally most parsimonious trees (score 63). All of the trees from both maximum likelihood and parsimony searches differ from one another only in the arrangement of sequences within haplotype group B.

Maximum likelihood bootstrap values are above nodes and maximum parsimony bootstrap values are below nodes.



## Discussion

The genotyping technique that we used here and in some of our previous work relies on a DNA recovery protocol with enhanced efficiency (Lalonde and Marcus 2019a). This has allowed us to recover rare mitochondrial DNA fragments that are larger than the ~200 bp mean fragment length recovered from many insect samples with degraded DNA (Heintzman *et al.* 2014; Tin *et al.* 2014) and use them as templates for successful PCR amplification, restriction digest-based genotyping, and high sensitivity capillary electrophoresis for the detection of diagnostic restriction fragment length polymorphisms (RFLPs) to determine mitochondrial haplotypes (Gemmell and Marcus 2015; Lalonde *et al.* 2018; Lalonde and Marcus 2019b). Here we have shown that the haplotype frequencies recovered from historical populations of *Junonia* butterflies are statistically indistinguishable from more contemporary populations collected from the same geographic regions, which is what we would expect if our method is effective and if haplotype frequencies in a given locality are stable over time. Except for cases where biological invasion events appear to have taken place, causing haplotype frequency fluctuations, haplotype frequencies appear to be stable in *Junonia* populations over many decades (Lalonde and Marcus 2019a).

The key advantage of this restriction-fragment-based genotyping method over other methods for mitochondrial haplotyping is its reliability. We were able to obtain mitochondrial haplotypes from all samples tested, including the oldest museum insect specimens genotyped to date (pre-1813, Table 2-3), while many earlier studies using other techniques for DNA isolation and mitochondrial genotyping from museum material failed to determine the haplotypes from a majority of the oldest insect specimens evaluated (Goldstein and DeSalle 2003; Keyghobadi *et al.* 2013; Heintzman *et al.* 2014). This facilitates the assembly of haplotype frequency data sets at the population-level with unprecedented resolution on both temporal and geographic scales, allowing us to ask a variety of interesting questions related to population genetics, invasion biology, and hybridization (Lalonde *et al.* 2018; Lalonde and Marcus 2019a; Lalonde and Marcus 2019b).

Many well-intentioned curators have used research results showing poor preservation of DNA preserved in museum specimens (Dean and Ballard 2001; Mandrioli *et al.* 2006; Espeland *et al.* 2010; Hernandez-Triana *et al.* 2014) to inform curatorial best practices (Wandeler *et al.* 2007). We have had the experience of having curators refuse to allow tissue collection for DNA extraction by destructive sampling from older specimens in their care because in their estimation, the likelihood of success would be low. Our findings in *Junonia* are consistent with other recent reports successfully recovering and genotyping DNA from 19$^{th}$ century insect material or from permafrost samples that may be thousands of years old (Goldstein and DeSalle 2003; Harper *et al.* 2006; Habel *et al.* 2009; Strange *et al.* 2009; Heintzman *et al.* 2014; Kanda *et al.* 2015; Prosser *et al.* 2016; Sproul and Maddison 2017), suggesting that these curatorial decisions were overly cautious and that new techniques for DNA recovery and genotyping can overcome the challenges of DNA degradation in preserved museum materials to provide valuable genotypic data.

The restriction-digest based approach to genotyping museum specimens is a high-throughput, low cost means of generating large data sets from historical material to generate large population genetic data sets spanning long periods of time. We have shown that this method is reliable and robust when used to genotype insect specimens collected at least as far back as the early 19$^{th}$ century and possibly earlier. The oldest preserved insect collections still extant are the James Petiver (1665-1718), Hans Sloane (1660-1735), and Joseph Banks (1743-1820) collections at the Natural History Museum, London (Stearns 1952; Hawkins 2010; Seaward 2010), the Carl Linneaus (1707-1778) collection at the Linnean Society of London (Tanner 1959; Honey and Scoble 2008), and the Kilian Stobaeus (1690-1742) collection at the Biological Museum of Lund University and the Queen Ludovica Ulrica (1720-1782) collection at the Evolution Museum of Uppsala University in Sweden (Kim and Lindroth 1978; Honey and Scoble 2008). Yet, preserved insect material from the 17$^{th}$ and even most of the 18$^{th}$ century is very scarce (Lalonde and Marcus 2019a). Thus, the vast majority of pinned insect specimens preserved in all museum collections were collected within the timeframe of the specimens included in our study.

While many collecting, storage, and curation practices in entomology are used across many orders of insects, others are taxon-specific. Historically, most butterfly specimens have been collected using hand-held aerial nets, killed by pinching or with one of many killing agents (ethyl acetate, ammonium carbonate, chloroform carbon tetrachloride, or potassium cyanide) and then dried and stored at room temperature in paper triangles or glassine envelopes prior to being mounted on a pin (Winter 2000). Unlike many other insect groups, the wings of Lepidoptera are usually spread during pinning, which often necessitates 24-48 hours of humidification in the presence of mold inhibitors (such as Captan, chlorocresol, chlorothalonil, formaldehyde, phenol, or thymol) (Winter 2000). Finally adult Lepidoptera are typically stored dry on pins, sometimes in the presence of fumigants (naphthalene, paradichlorobenzene, or Vapona (dichlorvos)) (Winter 2000). In spite of the fact that many of these conditions and substances act to degrade DNA in diverse ways (Grafstrom *et al.* 1983; Claycamp 1992; Makni *et al.* 2012), we were able to consistently recover mitochondrial genotypes from *Junonia* butterflies. We would expect that regardless of age, most insect specimens of similar size to New World *Junonia* (forewing length 20-26 mm (Peters and Marcus 2017)) and that were collected and preserved in a manner comparable to what is usually employed in processing Lepidoptera specimens would contain usable DNA for determining mitochondrial haplotypes and yield similar results to what we have seen here.

Future work should determine the limits and reliability of methods for obtaining nuclear genotypes from sets of historical specimens from a similar range of collection dates. There have been some reports of nuclear microsatellite loci failing to amplify from older museum material (Watts *et al.* 2007; Saarinen and Daniels 2012), especially those loci with long allele amplicons (Strange *et al.* 2009; Ugelvig *et al.* 2011). Yet, other studies have been very successful in recovering microsatellite markers (Harper *et al.* 2006; Habel *et al.* 2009) or Next-generation sequence data (Heintzman *et al.* 2014; Tin *et al.* 2014; Prosser *et al.* 2016; Sproul and Maddison 2017; Li *et al.* 2019) from 19$^{th}$ century material. This suggests to us that the difficulty of successfully obtaining nuclear genotypes from historical insect specimens is probably a surmountable methodological challenge, rather than a fundamental feature of this kind of material. With the right combination of techniques for DNA extraction, primer design and amplification

(focusing on small amplicons), and allele/sequence detection it may soon be possible to routinely characterize nuclear genetic variation from specimens of similar age to those for which we have recovered mitochondrial haplotypes.

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## Connecting section: Chapter 2 to Chapter 3

In Chapter 2, I validated a high throughput, low-cost method of mitochondrial genotyping using very old museum specimens, including some collected over 200 years ago. This allows for the reconstruction of historical changes in haplotype frequencies over hundreds of years. While developing this method, I obtained access to additional historical material for Florida *Junonia* species. These additional specimens will enhance our understanding of the current and historical distributions of the three *Junonia* species that occur in Florida. This is particularly important for the Tropical Buckeye (*Junonia zonalis*), a species which invaded from the Caribbean, as early records are limited. The additional sampling will allow me to compare the distribution and the genetic composition of contemporary and historical populations.

Chapter 3 will also address the origins of historical material attributed to Chokoloskee, Florida, a locality with many anomalous species records, leading some authors to question their validity. Comparing *Junonia* haplotype frequencies between Chokoloskee and possible source localities may help identify the geographic source of anomalous records from this and other species.

# Chapter 3: Back to the future: Updates on the invasion history of *Junonia* butterflies in Florida and the mystery of Chokoloskee.

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Author Contributions:

ML designed the study, did data collection, performed experiments, analyzed data and drafted the manuscript.

JM assisted with study design, provided laboratory space, research materials, and revisions to the manuscript.

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**Abstract**

The tropical buckeye, *Junonia zonalis* (Lepidoptera: Nymphalidae) is a recent addition to the butterfly fauna of mainland Florida. It appears that this species began to invade the mainland from the Florida Keys or Cuba by the 1930s, hybridizing with *J. coenia* and bringing with it mitochondrial haplotype group A, which is common in the Caribbean but is essentially absent from North American *Junonia*. By the 1940s, *J. zonalis* appears to have established populations in Miami, but eventually may have been extirpated. Later, new populations of *J. zonalis* may have become established on mainland Florida by subsequent waves of *J. zonalis* migrants. Substantial fluctuations in both population size and mitochondrial haplotype group A frequency seem to be characteristic of Florida mainland populations of *J. zonalis*. Populations of *J. zonalis* in the Florida Keys and Cuba have maintained nearly constant mitochondrial haplotype group A frequencies over many decades and may be more stable than those on the Florida mainland. *Junonia zonalis* specimens attributed to Chokoloskee, Florida, from the early 1900s have questionable provenance. Based on their haplotype frequency and other evidence these Chokoloskee specimens may have been collected in Cuba. Similarly, one specimen of *J. zonalis* likely collected during the 1880s and labeled "Indian River, Fla." probably also originated from outside of Florida.

## Introduction

The colonization of new habitats by non-native species is of concern to both conservationists and to those studying the effects of climate change (Falk-Petersen *et al.* 2006; Cristescu 2015). This process occurs continuously and understanding how both immigrant and resident populations interact and respond to this contact is one of the main focuses of invasion biology (Falk-Petersen *et al.* 2006; Cristescu 2015). In some cases, non-native colonists are referred to as invasive species, as they are organisms that have the potential to establish populations, adapt to new habitats, and displace native species (Didham *et al.* 2005; Falk-Petersen *et al.* 2006; Stigall 2010). Such species may have no natural predators, outcompete native species for habitat and resources, and/or hybridize with native species if reproductive isolation mechanisms are incomplete (Anderson and Hubricht 1938; Didham *et al.* 2005; Stigall 2010).

Historical specimens from natural history museum collections are an important resource for conservation and invasion biology studies (Goldstein and DeSalle 2003; Harper *et al.* 2006; Habel *et al.* 2009; Strange *et al.* 2009; Saarinen and Daniels 2012; Heintzman *et al.* 2014; Wells *et al.* 2015). These collections of organisms have been accumulated over space and time and may give insights into morphological and genetic variation between populations (Winston 2007). The development of digital specimen catalogs by many museums has greatly facilitated finding appropriate material for answering research questions. A series of influential studies have documented cases of "evolution in action" by making morphological comparisons at different time points using specimens from museum collections (Carroll *et al.* 2005; Miller-Struttmann *et al.* 2015). Similarly, a number of insightful studies have used museum specimens to examine changes in genetic variation over time within and between populations (Goldstein and DeSalle 2003; Harper *et al.* 2006; Habel *et al.* 2009; Strange *et al.* 2009; Heintzman *et al.* 2014; Wells *et al.* 2015). Yet, recovering genotypes from older museum specimens, especially from small species such as insects, has been very uneven due to technological limitations for isolating and genotyping DNA from minute tissue samples that were not originally collected and stored for this purpose (Saarinen and Daniels 2012). The most widely used methods for genotyping historical specimens have either employed Sanger sequencing of high copy number mitochondrial DNA or separation of different-sized

nuclear microsatellite alleles by electrophoresis (Goldstein and DeSalle 2003; Harper *et al.* 2006; Habel *et al.* 2009; Strange *et al.* 2009; Ugelvig *et al.* 2011; Saarinen and Daniels 2012; Keyghobadi *et al.* 2013; Heintzman *et al.* 2014), but many specimens fail to yield usable genotypes (Watts *et al.* 2007; Hernandez-Triana *et al.* 2014).

Recently, several advances in molecular technique have helped to resolve these limitations. Next-generation sequencing has been used by some researchers to acquire genotypes from older museum specimens (e.g. (Sproul and Maddison 2017; Cong *et al.* 2019)), but this technology is currently far more expensive on a per sample basis than the conventional Sanger sequencing and electrophoresis-based genotyping that it is replacing. By combining the advantages of high copy number mitochondrial based methods with the high sensitivity of fragment-based genotyping assays like those used for microsatellites, it has been possible to develop an extremely reliable, inexpensive, and high through-put method for obtaining genotypes from insect specimens in museum collections, including in some of the oldest ever genotyped (Lalonde and Marcus 2019a; Lalonde and Marcus 2019b; Lalonde and Marcus 2020). The method uses polymerase chain reaction to amplify small regions of the mitochondrial *cytochrome oxidase I* gene, which are then digested by restriction enzymes that cut at sites diagnostic for each alternative mitochondrial haplotype, and the resulting fragments are separated by highly sensitive and high-resolution capillary electrophoresis. To date, all (100%) of the over 1800 insect museum specimens that we have evaluated with this method (Gemmell and Marcus 2015; Lalonde *et al.* 2018; Lalonde and Marcus 2019b; Lalonde and Marcus 2019a; Lalonde and Marcus 2020) have yielded a mitochondrial haplotype, at a cost-per-sample that is similar to microsatellite genotyping, and 10–20% of the cost of Sanger sequencing, depending on the details of the specific restriction digest assay.

Here, we use this technique to expand on an earlier study investigating interactions between two resident species and one non-native invading species of buckeye butterfly (genus *Junonia* Hübner, Lepidoptera: Nymphalidae) in South Florida, USA (Lalonde and Marcus 2019b). This genus was an ideal candidate for historical study as only two principle mitochondrial haplotype groups exist in Florida (haplotype groups A and B; 4% sequence divergence between them) and both haplotypes are shared between virtually all *Junonia* species in the Western Hemisphere (Peters and Marcus

2017). Haplotype group B occurs at nearly 100% frequency in North America, Mexico and Central America, while haplotype group A predominates in South America (Pfeiler *et al.* 2012; Borchers and Marcus 2014; Gemmell and Marcus 2015). The Caribbean appears to be a region of genotype admixture, but each Caribbean Island appears to have characteristic mitochondrial haplotype frequencies that are shared by all of the species of *Junonia* found on that island (Gemmell *et al.* 2014; Gemmell and Marcus 2015). Three *Junonia* species occur in Florida: *J. coenia* Hübner (common buckeye), *J. neildi* Brévignon (mangrove buckeye), and *J. zonalis* C. & R. Felder (West Indian or tropical buckeye) (Lalonde *et al.* 2018).

All three species are well represented in natural history collections, and the invasion of the non-native species (*J. zonalis*) is well documented (Lalonde *et al.* 2018; Lalonde and Marcus 2019b). The earliest specimen of *J. zonalis* known with reliable locality data from South Florida was collected in 1961 (Calhoun 2010), but a number of suspected hybrids (mostly *J. zonalis* × *J. coenia*) from as early as the 1930s have also been identified (Lalonde and Marcus 2019b), suggesting that the initial *J. zonalis* invasion may have been earlier. Two additional *J. zonalis* specimens described as originating from Chokoloskee, Collier County, Florida from the early 1900s were also examined and genotyped, but it has been suggested that many insect specimens attributed to this locality during this period were actually collected elsewhere (Young 1955; Kimball 1965; Heppner 1993), thus the exact timing of the first arrival of *J. zonalis* in Florida has remained an open question (Lalonde *et al.* 2018).

The problematic records from Chokoloskee are associated with material from a collector/dealer who is referred to as "Mrs. C.G. McKinney" when mentioned by name in the literature, who lived in Chokoloskee from 1886 to 1926 (Blatchley 1932). McKinney, whose maiden name was Martha Susan Starling, was the second wife of the postmaster of Chokoloskee, Charles Greenleigh McKinney. Chokoloskee is a community at the northern edge of the Everglades in southwest Florida, which consisted of ten families in 1912 (Tebeau 1955). Specimens provided by McKinney are rarely labeled with her name and she is often unnamed in the entomological literature (e.g. she is described only as "our collector in Chokoloskee, Fla." by Barnes & McDunnough (1916)), but she provided numerous Lepidoptera, Coleoptera, and Orthoptera specimens to collectors and

dealers (Kimball 1965). This material is usually associated with Chokoloskee, but a subset is labeled as originating from Marco or Everglade (now Everglade City), perhaps because some shipments of McKinney's specimens had postmarks from those locations (Tebeau 1955; Kimball 1965). Lepidopteran material collected by McKinney (perhaps with the assistance of other members of the Chokoloskee community) is particularly well represented in the William Barnes collection, now part of the Smithsonian National Museum of Natural History (Barnes and McDunnough 1916), but it also appears in other collections (Smith 1908: 119, Grossbeck 1917: 12, Todd 1982: 77).

Based on morphological subspecies differences between Florida and elsewhere in the Neotropics, some of the McKinney's insect specimens attributed to Chokoloskee, Marco, or Everglade may actually originate from either Central/South America or the West Indies (Grossbeck 1917: 8, Klots 1951: 283, Young 1955, Kimball 1965, Heppner 1993, Woodruff 2004), resulting in erroneous or dubious Florida records from the first two decades of the 20$^{th}$ Century (Calhoun 1997). Since there are species names (mostly junior synonyms) with Chokoloskee type localities based on this material, it would be valuable to determine the true geographic origins of such specimens (e.g. Smith 1908: 119, Leng 1915). During this period, large numbers of Americans, perhaps including young men from Chokoloskee, were serving in Cuba (1898–1909) and Puerto Rico (beginning in 1898) after the Spanish-American War, and in Panama during the construction of the Panama Canal (1903–1914). These localities represent particularly likely sources of foreign specimens that might have been sent to McKinney, and which eventually became attributed to Chokoloskee after she distributed them to buyers in the United States.

In our prior work (Lalonde and Marcus 2019b), we determined that the haplotype frequencies of *J. zonalis* in the lower Florida Keys are nearly identical to those found in Cuba and the Bahamas (~35% A, ~65% B), which is perhaps consistent with ongoing genetic exchange between those populations. Some authors have considered Cuba to be the more likely source of migrants due to its geographic proximity to the lower Florida Keys populations of *J. zonalis* (Minno and Emmel 1993; Cech and Tudor 2007). Florida mainland populations of *J. zonalis* show much lower and rather variable frequencies of haplotype A (<10% A, which is otherwise almost completely absent in all *Junonia* found

on mainland North America) than are found in the Florida Keys, Cuba, the Bahamas, or elsewhere in the Caribbean, and may only experience episodic genetic exchange with other *J. zonalis* populations. The first documented occurrence of *Junonia* haplotype group A in Florida is from a *J. coenia* specimen collected in 1934, suggesting like the presence of *J. zonalis* hybrids during the 1930s, and that genetic exchange with individuals from the Caribbean occurred prior to that date.

Since our original work (Lalonde and Marcus 2019b), we have collected additional *Junonia* material in the field and visited several additional museum collections (particularly the American Museum of Natural History (AMNH), the Smithsonian National Museum of Natural History (USNM), and the Oxford University Museum of Natural History (OUMNH)), where we discovered *Junonia* specimens from South Florida and Cuba that fill important temporal gaps in our original report. In particular, these include new (2019) field collections of *J. zonalis* from southwestern Broward County Florida, *J. coenia* and *J. neildi* museum material from Cuba (OUMNH) which was not well represented in our earlier publications, a 1940s series of *J. zonalis* specimens from Miami, Florida collected by Otto Buchholz (1874–1958) (AMNH), a *J. zonalis* specimen from "Indian River, Fla." from the Berthold Neumoegen (1845–1895) collection (USNM), and an expanded early 1900s series of *J. zonalis* specimens from Chokoloskee, Florida (preserved in the William Barnes Collection, now at USNM).

We have carefully examined this additional material and obtained mitochondrial haplotypes in order to increase the temporal resolution of the invasion history of *J. zonalis* and to reexamine hypotheses about whether the invasion has been continuous or episodic. The analysis presented here provides additional detail regarding the formation of the secondary contact zone between *J. zonalis* and the other two *Junonia* in Florida. We also have an opportunity to try to clarify the geographic origins of insect specimens attributed to Chokoloskee, Florida by comparing haplotype frequencies from McKinney's *Junonia* series from the early 20$^{th}$ century with haplotype frequencies from *Junonia* populations elsewhere. In doing so, if one assumes that all misattributed material comes from the same location, it may be possible to exclude some localities as the source of questionable Chokoloskee insect material from the early 20$^{th}$ century (not just *Junonia*)

(Young 1955; Kimball 1965; Heppner 1993) and to identify those localities that most closely match mitochondrial haplotype frequencies found in Chokoloskee *Junonia*.

## Materials and Methods

**Specimen Collection and Preparation**. A total of 1023 specimens were selected based on geographic location (south Florida, Cuba, Bahamas, Panama, Puerto Rico), date of collection, and morphology-based species identification: common buckeye (*J. coenia*), Caribbean mangrove buckeye (*J. neildi*), tropical/West Indian buckeye (*J. zonalis*), and the South American mangrove buckeye (*J. litoralis* Brévignon, 2009; Panama only). Specimens were identified on the basis of morphological characters as previously described (Lalonde *et al.* 2018; Lalonde and Marcus 2019b). Many recent specimens were collected by members of our laboratory, some which have been described previously (Gemmell and Marcus 2015; Lalonde *et al.* 2018; Lalonde and Marcus 2020). To supplement what we collected ourselves geographically and chronologically, we borrowed additional specimens from both private collectors and museum collections (Electronic Supplement 3, https://mspace.lib.umanitoba.ca/xmlui/handle/1993/34536). In total, specimens from 16 natural history museum collections (AMNH, C.P. Gillette Museum of Arthropod Diversity at Colorado State University, California Academy of Sciences, Canadian National Collection of Insects, Arachnids, and Nematodes, Harvard University Museum of Comparative Zoology, Illinois Natural History Survey Prairie Research Institute at the University of Illinois at Urbana-Champaign, LSA Museum of Zoology at the University of Michigan, McGuire Centre for Lepidoptera and Biodiversity at the Florida Museum of Natural History (MGCL), Albert J. Cook Arthropod Research Collection at Michigan State University, Milwaukee Public Museum (MPM), Mississippi Entomological Museum at Mississippi State University, Oregon State Arthropod Collection Corvallis at Oregon State University, USNM, J.B. Wallis / R. E. Roughley Museum of Entomology at the University of Manitoba, Yale Peabody Museum of Natural History, and Oxford University Museum of Natural History) and six private collections (John Calhoun, Charles Covell, Carlos Cruz, Jeff Slotten, Marc Minno, and the Marcus Lab) were included in this study.

Specimen DNA was isolated from a single butterfly leg with the Qiagen DNEasy Blood and Tissue kit where the kit lysis buffer was replaced with "mouse tail-tip" lysis buffer as previously described (Lalonde and Marcus 2019b). Extractions were performed manually or with the assistance of a Qiagen QIAcube extraction robot (Qiagen, Düsseldorf, Germany) using the Animal tissue DNA program, following the manufacturers protocol with modifications as previously described (Gemmell and Marcus 2015). Sample DNA concentrations were evaluated using a Nanodrop 2000 spectrophotometer (Nanodrop, Wilmington, Delaware, USA) and subsequently stored at -20˚C until use.

**Mitochondrial *Cytochrome c Oxidase I* (*COI*).** Fragments of *cytochrome c oxidase subunit I* (*COI*) were amplified by polymerase chain reaction (PCR). Primers are given in Table 3-1. PCR reaction conditions used were as follows: 95˚C for 5 minutes; 35 cycles of 94˚C for 1 minute, 46˚C for 1 minute, 74˚C for 1 minute, 94˚C for 1 minute; and a final extension for 5 minutes at 72˚C, then a 4˚C hold. Visualization of amplification products from samples were run on a QIAxcel Advanced capillary electrophoresis instrument (Qiagen) as reported previously (Gemmell and Marcus 2015). Negative control amplifications with distilled deionized water (instead of extracted DNA) were included within each PCR reaction. Whenever amplification was detected in the negative controls, all PCR reactions from the experiment were discarded and subsequent PCR amplification was repeated with fresh reagents. These protocols for PCR and visualization of PCR products and restriction digests were used for all experiments described here, unless otherwise specified. If no PCR products were obtained from the LCO1490/ HCO2198 amplification of samples, they were reamplified using primers that produced smaller amplification products (Table 3-1).

**Table 3-1.** PCR Primer names, sequences, and sizes.

| Primer Name | Primer Sequence | PCR Product Size |
|---|---|---|
| LCO1490 (f)<br>(Folmer *et al.* 1994) | GGT CAA CAA ATC ATA AAG ATA TTG G | 709 bp<br>(with primers) |
| HCO2198 (r)<br>(Folmer *et al.* 1994) | TAA ACT TCA GGG TGA CCA AAA AAT CA | 658 bp<br>(primers removed) |
| miniCOIF2 (f)<br>(Gemmell and Marcus 2015) | ATA CTA TTG TTA CAG CCT CAT GC | 569 bp<br>(with primers) |
| HCO2198 (r)<br>(Folmer *et al.* 1994) | TAA ACT TCA GGG TGA CCA AAA AAT CA | 520 bp<br>(primers removed) |
| miniCOIF2 (f)<br>(Gemmell and Marcus 2015) | ATA CTA TTG TTA CAG CCT CAT GC | 501 bp<br>(with primers) |
| miniCOIR3 (r)<br>(Gemmell and Marcus 2015) | TAT TTC GAT CTG TTA AAA GTA TAG | 454 bp<br>(primers removed |

If adequate PCR products were identified, a diagnostic restriction enzyme digest was performed using AflIII, and BamHI restriction endonucleases (New England Biolabs (NEB), Ipswich, MA, USA) to unambiguously identify the haplotype group of each specimen as previously described (Gemmell and Marcus 2015; Lalonde *et al.* 2018; Lalonde and Marcus 2020). The digested products were resolved using a QIAxcel Advanced instrument as described above for evaluating PCR products.

**Haplotype Frequency Changes in Space and Time.** Samples were sorted by species, collection locality, and year of collection. A small number of specimens that lacked collection dates from the Gemmel & Marcus (2015) data set were excluded from analyses using date of collection. For each locality and collection year, the total numbers of haplotype A and haplotype group B were tallied for each species.

Haplotype frequency graphs were generated using Microsoft Excel (Redmond, WA, USA) using the proportion of haplotype A for each species. Data sets were sorted by place (mainland Florida, the Florida Keys, Cuba, and Bahamas), by species, and then by year. The proportion of haplotype A for each decade (e.g., 1980–1989) was then calculated and plotted. Standard error for each percentage was calculated using standard methods by taking the square root of ((proportion A*(1-proportion A))/total sample size) for each decade (Stuart 1963). The standard error bars created are a function of both the proportion of haplotype A and the total sample size for each decade, therefore data points with the same sample size may have different standard error values due to differing proportions of haplotype A.

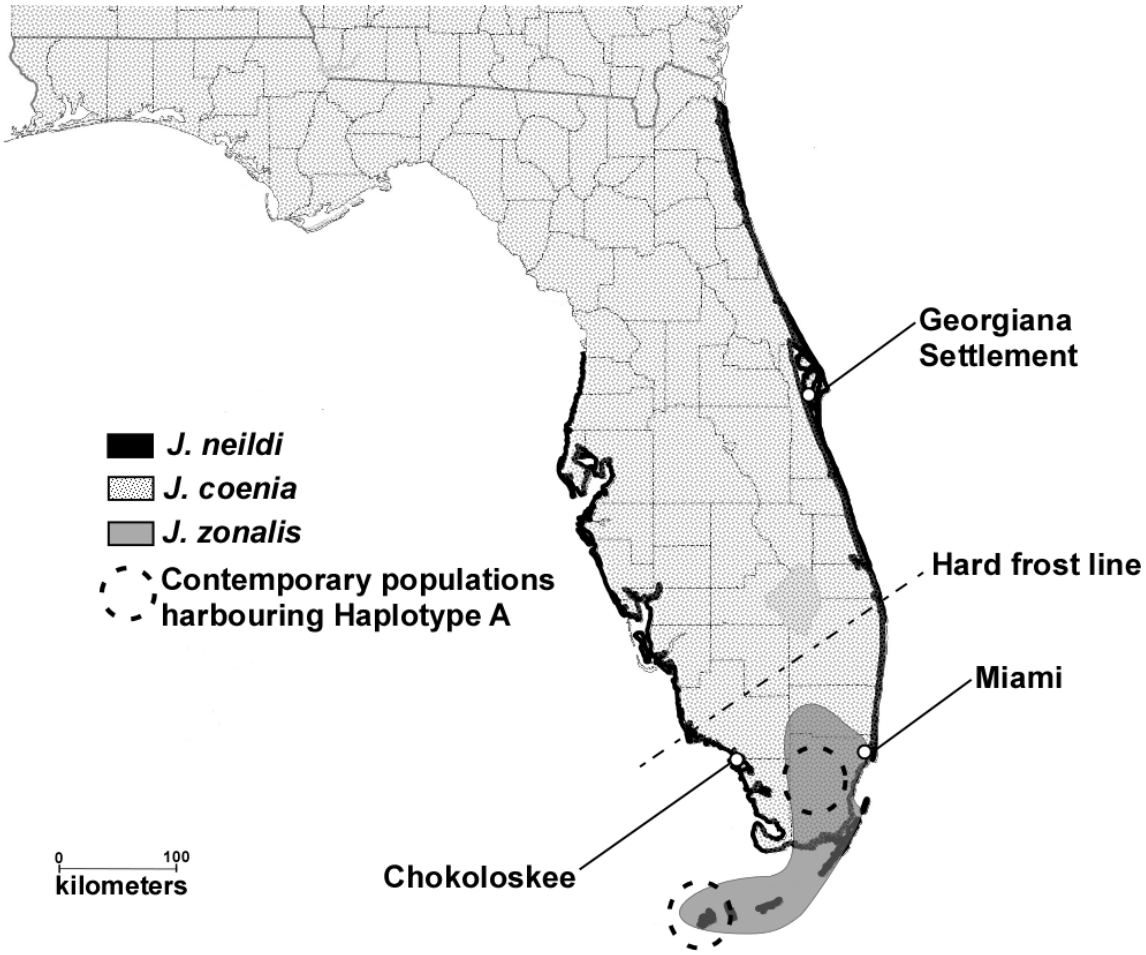
**Provenance of Chokoloskee *Junonia* Specimens.** To begin to make inferences about the likely provenance of *Junonia* specimens from Chokoloskee, we compared the mitochondrial haplotype frequencies of *J. zonalis* specimens collected during the early 20$^{th}$ century and attributed to Chokoloskee, Florida from four museum collections (AMNH, USNM, MGCL, and MPM). We compared these samples with *J. zonalis* samples collected from elsewhere in mainland Florida, Cuba, Puerto Rico, and Panama. DNA was isolated and mitochondrial haplotypes were determined as described, above and mitochondrial haplotype frequencies were estimated for each population. To avoid possible misinterpretations due to the presence of *J. zonalis* hybrids in the comparison groups, for localities where putative hybrids were observed, we made the comparisons both with and without these hybrids.

## Results

A total of 1025 specimens from South Florida (737), Cuba (159), the Bahamas (52), Panama (50), and Puerto Rico (27) spanning the years 1866–2017 were evaluated and genotyped, including 348 *J. coenia,* 321 *J. neildi*, 303 *J. zonalis,* 5 *J. litoralis*, and 48 hybrids. Figure 3-1 updates the distributions of *Junonia* species in Florida, based on additional contemporary and historical sampling. The range of *J. zonalis* extends farther north and east than was previously recognized, including parts of Monroe and Broward Counties and nearly all of Dade County. The contemporary Florida populations harboring

haplotype group A identified by to our previous work are also indicated (Lalonde and Marcus 2019b).

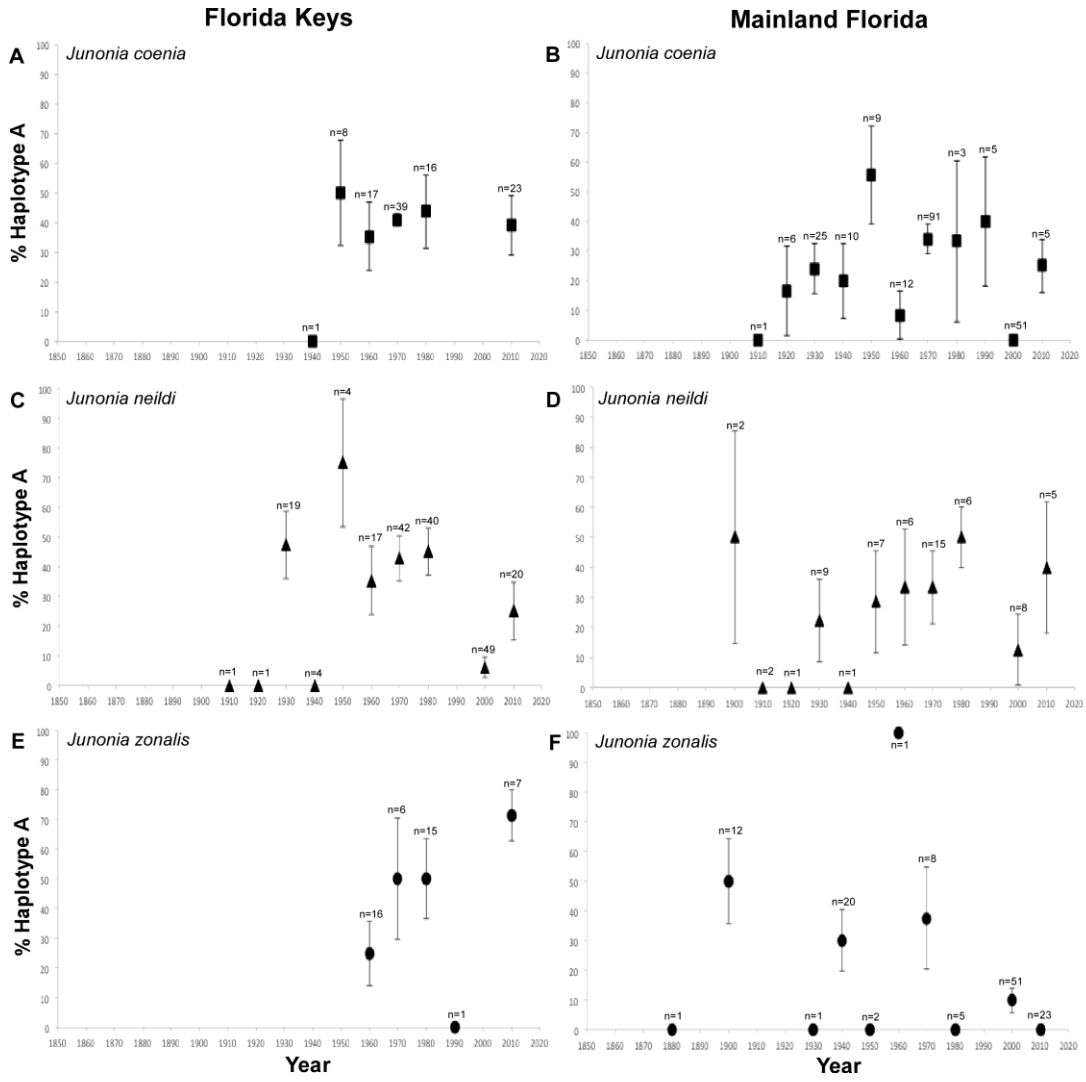
**Figure 3-1.** Updated ranges of *Junonia* species in Florida. Revised from Lalonde and Marcus 2019b to reflect additional localities for *J. zonalis* identified from museum specimens and from recent field collections. Contemporary (2000–2019) Florida populations harboring haplotype group A are circled.




**Temporal patterns of *Junonia* haplotypes.** *Junonia coenia* specimens from the Florida Keys are found in museum collections beginning in the 1940s with a consistent proportion of haplotype A over time of ~40% (Figure 3-2 (A)). *Junonia coenia* from mainland populations are represented in collections in sizeable numbers starting in the

1920s, and the number of available specimens available for study has increased during the subsequent decades (Figure 3-2 (B)). The frequency of haplotype A seems to oscillate over time (Figure 3-2 (B)): from the 1920s until the 1940s the haplotype was ~30%, while during the 1950s haplotype A frequencies increased to ~60% haplotype, followed by a decrease to ~10% during the 1960s. From the 1970s until the 1990s a consistent frequency ~40% haplotype A was observed. During the 2000s decade, the haplotype frequency crashed to 0% and in the current decade based on few samples, a potential recovery may be taking place.

**Figure 3-2.** Comparison of the Florida Keys and Mainland Florida *Junonia* species using the proportion of haplotype A (± 1 standard error (SE)) over time in Florida, USA: (**A**) *Junonia coenia* Florida Keys, (**B**) *Junonia coenia* Mainland Florida, (**C**) *J. neildi* Florida Keys, (**D**) *J. neildi* Mainland Florida, (**E**) *J. zonalis* Florida Keys (including *J. zonalis* hybrids), (**F**) *J. zonalis* Mainland Florida (including *J. zonalis* hybrids).




Few *J. neildi* specimens from the Florida Keys were available for study prior to the 1930s (Figure 3-2 (C)). The frequency of haplotype A also seems to oscillate over time (Figure 3-2 (C)). The frequency of haplotype A during the 1930s is ~45% which is followed by a drop down to 0% during the 1940s. The 1950s display a haplotype frequency of ~75%, though, this is based a small sample size. The 1960s to the 1980s show haplotype frequencies that increase from ~35% to ~45%. There were no *J. neildi* samples from the 1990s available for sampling from the Florida Keys. During the 2000s the haplotype frequency was less than 10% and in the present decade there seems to be an increase to ~25%.

The earliest *J. neildi* specimens from mainland Florida found in the collections that were available for study were from the early 1900s (Figure 3-2 (D)). The frequency of haplotype A from the 1930s until the 1980s remained relatively constant between ~25% and ~40%. There were no samples of *J. neildi* available for the 1990s. During the 2000s the frequency of haplotype A dropped to ~15% and in the present decade increased to ~40%.

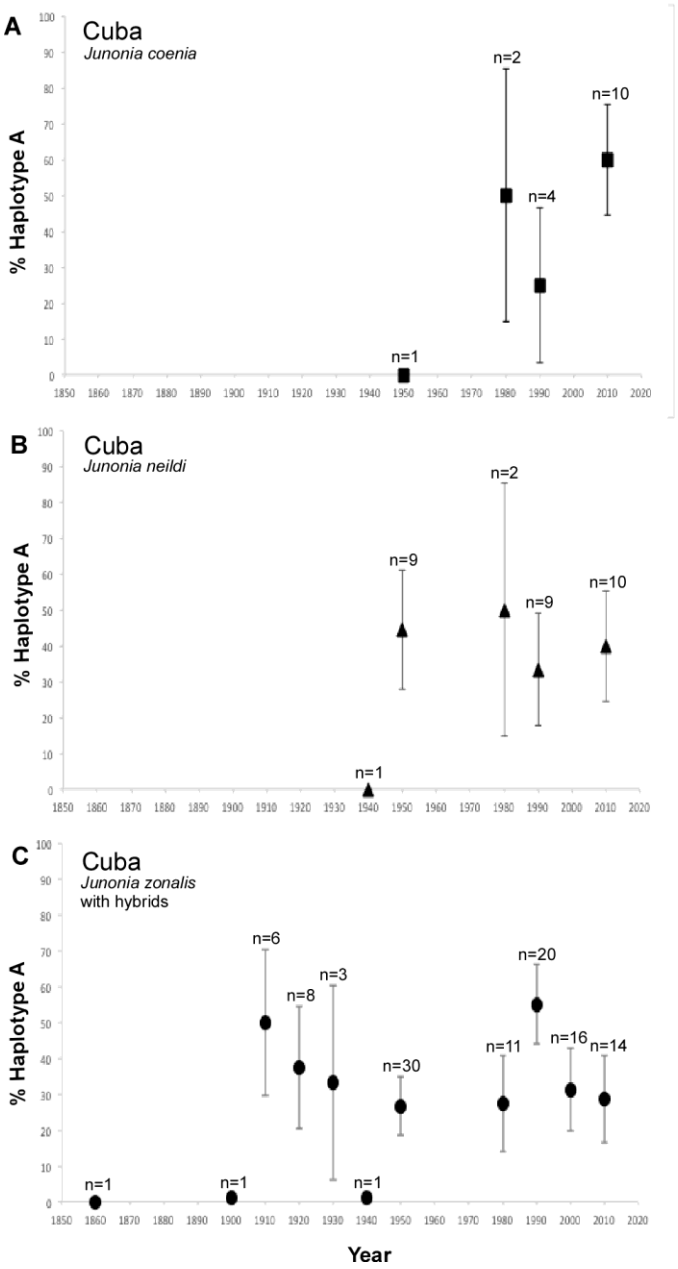
*Junonia zonalis* was not detected in the Florida Keys until the 1960s (Figure 3-2 (E)). As previously reported (Lalonde and Marcus 2019b), suspected *J. zonalis* hybrids were identified in Florida and were also included in our data sets. The frequency of haplotype A for *J. zonalis* and suspected hybrids was ~25% during the 1960s and increased to ~50% during the 1980s. A single sample from the 1990s was obtained, which carried haplotype B. *Junonia zonalis* samples from the 2000s were not available for genotyping from the Florida Keys. During the 2010s, the haplotype A frequency of *J. zonalis* in the Florida Keys was ~70% though this is based on a small sample (n=7; Figure 3-2 (E)).

The earliest *J. zonalis* attributed to mainland Florida is labeled “Indian River” and was probably collected during the 1880s (Figure 3-2 (F), see discussion). During the 1900s, the series of samples from Chokoloskee were found to have a haplotype A frequency of ~50% (Figure 3-2 (F)). No samples were available for genotyping from the 1910s, or the 1920s, and only a single probable hybrid individual was available from the 1930s. The *J. zonalis* collected during the 1940s from mainland Florida have a haplotype group A frequency of ~30%, which by the 1970s had increased to ~40%. The 1980s *J. zonalis* specimens from the Florida mainland have a haplotype A frequency of 0%. No samples were obtained for the 1990s for *J. zonalis* from mainland Florida. During the 2000s, the frequency of haplotype A in mainland *J. zonalis* was found to be ~10% and then drops to 0% in the 2010s.

Of the three *Junonia* species found in Cuba, *J. coenia* is represented in the fewest specimens (Figure 3-3 (A)). We obtained a single specimen from the 1950s, two specimens from the 1980s and four from the 1990s. Because of these small sample sizes, haplotype frequencies are prone to sampling error for these decades. For the 2010s we were able to obtain ten specimens which yielded a 60% haplotype A frequency. There

were more *J. neildi* samples available for sampling (Figure 3-3 (B)), but only three decades had large enough sample sizes to make reasonable calculations of haplotype frequencies. The 1950s, 1990s, and 2010s had haplotype A frequencies between ~35% and ~50% (Figure 3-3 (B)). *Junonia zonalis* is the most abundant *Junonia* species in Cuba (Figure 3-3 (C)). From the 1910s onward, sizeable numbers of specimens were collected, though two specimens with earlier collection dates were found (Figure 3-3 (C)). In contrast to the patterns seen on mainland Florida, the frequency of haplotype A in Cuba has remained relatively constant throughout the sampled time interval, at ~40% (Figure 3-3 (C)).

**Figure 3-3.** Proportion of haplotype A (± 1 standard error (SE)) over time for *Junonia* species from Cuba: (**A**) *J. coenia*, (**B**) *J. neildi*, (**C**) *J. zonalis* (including *J. zonalis* hybrids).




**Chokoloskee *Junonia zonalis.*** In an attempt to identify where the problematic Chokoloskee specimens may have originated, we compared *J. zonalis* populations from mainland Florida, Florida Keys, Cuba, Puerto Rico, and Panama (Figure 3-4). While

different populations showed distinct haplotype frequencies, the modest number of *J. zonalis* specimens from Chokoloskee (n=13) unfortunately means that the 95% confidence intervals (=2 standard errors) remain quite large and overlap with the confidence intervals of all candidate source populations (Table 3-2). Consequently, we cannot statistically exclude any of the candidate geographic localities, but we can say that the point estimates for the *J. zonalis* haplotype A frequency from Chokoloskee of 41.7 to 46.2% are much more similar to the point estimates for haplotype A frequencies for Cuba (36.0 to 37.5%), Puerto Rico (38.1%), and the Florida Keys (44.4 to 50.0%) than they are to the haplotype A frequencies for Panama (24.4%) or *J. zonalis* collected elsewhere on the Florida mainland (15.0 to 16.9 %) (Table 3-2).

**Figure 3-4.** Comparison of *Junonia zonalis* populations using the total proportion of haplotype A (± 1 standard error (SE)) for all time points for Florida, USA (Chokoloskee, mainland, and Keys), Cuba, Puerto Rico, and Panama.

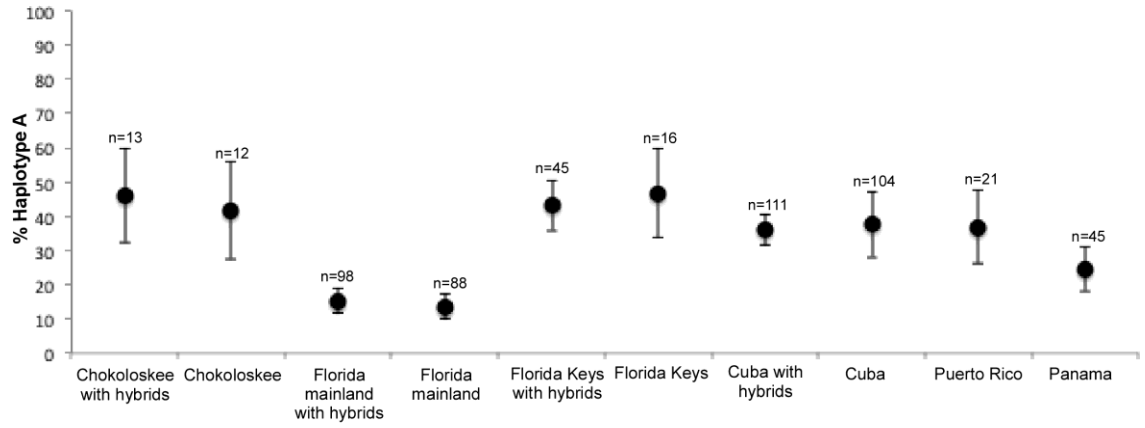


**Table 3-2.** Frequencies of mitochondrial haplotype group A in *Junonia* butterflies for different regions and species.

| Region | Species | Sample Size | Proportion A | 1 SE | 2 SE |
|---|---|---|---|---|---|
| Florida – mainland | *J. coenia* | 218 | 0.229 | 0.028 | 0.057 |
| | *J. neildi* | 62 | 0.290 | 0.058 | 0.115 |
| | *J. zonalis* | 113 | 0.150 | 0.034 | 0.067 |
| | *J. zonalis* including hybrids | 124 | 0.169 | 0.034 | 0.067 |
| Florida - Keys | *J. coenia* | 104 | 0.404 | 0.048 | 0.096 |
| | *J. neildi* | 197 | 0.315 | 0.033 | 0.066 |
| | *J. zonalis* | 16 | 0.500 | 0.125 | 0.250 |
| | *J. zonalis* including hybrids | 45 | 0.444 | 0.074 | 0.148 |
| Florida - Chokoloskee | *J. neildi* | 12 | 0.167 | 0.108 | 0.215 |
| | *J. neildi* including hybrids | 13 | 0.231 | 0.117 | 0.234 |
| | *J. zonalis* | 12 | 0.417 | 0.142 | 0.285 |
| | *J. zonalis* including hybrids | 13 | 0.462 | 0.138 | 0.277 |
| Bahamas | *J. coenia* | 10 | 0.400 | 0.155 | 0.310 |
| | *J. neildi* | 25 | 0.280 | 0.090 | 0.180 |
| | *J. zonalis* | 17 | 0.412 | 0.119 | 0.239 |
| Cuba | *J. coenia* | 17 | 0.471 | 0.121 | 0.242 |
| | *J. neildi* | 31 | 0.387 | 0.087 | 0.175 |
| | *J. zonalis* | 104 | 0.375 | 0.047 | 0.095 |
| | *J. zonalis* including hybrids | 111 | 0.360 | 0.046 | 0.091 |
| Puerto Rico | *J. neildi* | 6 | 0.167 | 0.152 | 0.304 |
| | *J. zonalis* | 21 | 0.381 | 0.106 | 0.212 |
| Panama | *J. zonalis* | 45 | 0.244 | 0.064 | 0.128 |
| | *J. litoralis* | 5 | 0.200 | 0.179 | 0.358 |

## Discussion

Cuban *J. zonalis* was well represented in our original data set but the sampling of *J. coenia* and *J. neildi* from Cuba was limited (Lalonde and Marcus 2019b). Additional sampling of all three species from Cuba has allowed for a more thorough understanding of *Junonia* mitochondrial haplotype frequencies in Cuba and the Caribbean. We can now confirm that the frequency of Caribbean haplotype group A alleles in Cuban *J. zonalis* is

stable at ~40% as we previously reported (Lalonde and Marcus 2019b) and that this frequency is stable and consistent across all three species in Cuba (Figure 3-3).

**Revising the invasion history of Florida *Junonia*.** Lalonde and Marcus (2019b) suggested based on statistical analyses of haplotype frequency data, that Cuba is the most likely source of *J. zonalis* and the Caribbean haplotype group A alleles that have been observed in Florida. The haplotype frequencies in Cuba most closely matched those in Key West. Although the Bahamas is another possible source population, the frequencies did not match the frequencies found in the northern populations of the Florida Keys, for example from Key Largo (Lalonde and Marcus 2019b). Based on additional sampling, Cuba still seems to be the most likely candidate for *J. zonalis* and the A allele in the Florida Keys (Figure 3-2 (F), Figure 3-3 (C), Figure 3-4.).

Initially, we and others speculated that *J. zonalis* arrived in South Florida soon before 1961 based on the single "index case" specimen that was collected in that year and was later discovered in the collection of the University of Michigan Museum of Zoology (Cech and Tudor 2005; Calhoun 2010; Lalonde *et al.* 2018). Then, Lalonde and Marcus (2019b) subsequently found a number of probable *J. zonalis* hybrids from mainland Florida from as early as the 1930s in museum collections, and also recorded the presence of the Caribbean haplotype group A in mid-20$^{th}$ Century *J. coenia* specimens (which based on contemporary work were otherwise only known to carry haplotype group B). This suggested that *J. zonalis* and the Caribbean allele were present in Florida much earlier than originally thought. As we have reported here, with further investigation of several additional museum collections, we have been able to locate additional specimens that, based on morphology, are clearly assignable to *J. zonalis* that were collected by Otto Buchholz from what appears to be a single location in Miami over a period of several years during the 1940s. This, plus our own collections of *J. zonalis* from southwestern Broward County in 2019 extends the known range of *J. zonalis* from what was reported by (Lalonde and Marcus 2019b) to the north and east to include most of Miami-Dade County and the southwestern corner of Broward County (Figure 3-1). Among the *J. zonalis* collected by Buchholz is one additional specimen with an Orlando, Florida, 6 October 1945 collection label. This may be an additional Miami specimen with an

erroneous locality label, or it may represent the capture of a rare stray outside of the documented typical range of *J. zonalis* in Florida. Similar stray individual *J. zonalis* were collected in Central Florida during the 2000s: one at Payne's Prairie (Alachua County) and one in Orlando (Orange County) (Lalonde *et al.* 2018).

These findings further support the hypothesis of Lalonde and Marcus (2019b) that *J. zonalis* and mitochondrial haplotype group A from the Caribbean were present in Florida long before 1961 and probably since the 1930s. The relatively stable haplotype group A frequencies over time in *J. zonalis* from the Florida Keys (Figure 3-2 (E)) are in contrast to the fluctuating haplotype group A frequencies on the Florida mainland (Figure 3-2 (F)), suggesting that the population dynamics of Florida mainland *J. zonalis* are qualitatively different from those of the Keys. One of the drivers of these fluctuations may be the periodic migration of *J. zonalis* from Cuba or the Florida Keys to the Florida mainland, as has been suggested previously (Cech and Tudor 2005; Lalonde and Marcus 2019b).

***Junonia* and the mystery of Chokoloskee.** There is a long history of skepticism in the entomology community with respect to early 20$^{th}$ century insect material attributed to the locality of Chokoloskee, Florida (Grossbeck 1917: 8, Klots 1951: 283, Young 1955, Kimball 1965, Heppner 1993, Woodruff 2004). Many species or subspecies purportedly collected there and sold by Martha McKinney are otherwise absent from Florida (and the rest of North America), and when other collectors visited Chokoloskee they were unable to find the same rare species and confirm the records (Blatchley 1932; Kimball 1965; Heppner 1993). Somehow "South American and West Indian species" (Young 1955) were being sold as originating from Chokoloskee and nearby locations (Everglade City, Marco Island) in Florida, either because Martha McKinney was reshipping material that she had received from elsewhere (Young 1955), or because American dealers handling her specimens added foreign material to what she had collected before resale (Kimball 1965). Because few other collection localities have experienced the degree of specimen misattribution as Chokoloskee, we believe it is more likely that it was the labeling practices of McKinney herself, rather than others handling her material that has produced this phenomenon. We strongly suspect that she had a correspondent, perhaps a young man from the small close-knit Chokoloskee community (Tebeau 1955), who was able to

collect and send material while he was working on the construction of the Panama Canal, or while he was stationed with the American military in Cuba or Puerto Rico after the Spanish-American War. In our earlier work, we included two Chokoloskee *Junonia* specimens, but we treated those records as questionable (Lalonde and Marcus 2019b). We hoped that by genotyping larger numbers of early 20$^{th}$ century *Junonia* from Chokoloskee, we could distinguish between some of these alternatives.

The mitochondrial haplotype group A frequency for *Junonia* specimens attributed to Chokoloskee was estimated to be between 41.7 to 46.2%, depending on whether possible hybrids are included in the calculation (Table 3-2). This makes them very dissimilar to the haplotype frequencies found elsewhere in mainland Florida (15.0 to 16.9%), suggesting that like many other specimens from Chokoloskee, they were collected in another locality and are mislabeled. Panama also has a very dissimilar haplotype A frequency (24.4%) and so is an unlikely source of this material. Haplotype group A frequencies from Cuba (36.0 to 37.5%), Puerto Rico (38.1%), and the Florida Keys (44.4 to 50.0%) are similar to one another and to those from Chokoloskee, they are the more likely sources of the Chokoloskee material (Figure 3-4). Unfortunately, because of the modest sample size of *Junonia* from Chokoloskee (N=13, N=14 with hybrids), the 95% confidence interval around the haplotype frequency estimate is large, so we cannot distinguish between these alternatives. A number of other insect species misattributed to Chokoloskee on the basis of McKinney's specimens are common in Cuba (e.g. *Euptoieta hegesia* (Cramer) (Nymphalidae), *Eurema messalina* (Fabricius) (Pieridae), *Phoebis argante* (Fabricius) (Pieridae), *Heraclides aristodemus temenes* (Godart) (Papilionidae) (Calhoun 1997; Hernandez 2004)) or are Cuban endemics (e.g. *Eurytides celadon* (Lucas) (Papilionidae) (Heppner 1993), *Alaus patricius* (Candeze) (Coleoptera: Elateridae)) (Schaeffer 1909), thus we believe that Cuba is the most likely source of much of the McKinney's material that is misattributed to Chokoloskee.

**"Indian River" *Junonia zonalis*, the earliest of all?** The "Indian River, Fla." specimen of *J. zonalis* from the Neumoegen collection (FLG1900.2, USNM) is not labeled with a collector's name or collection date, but this is typical of material labeled with this designation from William (Wilhelm) Wittfeld (1828-1913), who collected Lepidoptera at

Georgiana, Brevard County, Florida between 1880 and 1892 (Calhoun 1994; Calhoun 1997). Especially during the early part of this interval, Wittfeld sent thousands of Lepidoptera specimens to William H. Edwards (1822-1909), who then redistributed some of it to Berthold Neumoegen and Henry Edwards (1827-1891) (Calhoun 1994), thus this specimen likely dates to the early 1880s, and may be the earliest still-extant specimen of *J. zonalis* from Florida. However, this specimen has a particularly wide white postmedian band on the ventral hindwing, which is unusual for Florida *J. zonalis* (and is more typical of *J. zonalis* material from the Caribbean or Central America) and only very rare strays of *J. zonalis* have been found as far north as Brevard County, Florida (in Orlando and Paynes Prairie, as discussed above). Neumoegen, who had among the largest collections in North America, also received tens of thousands of specimens from diverse other localities during this period (Leach 2013), thus it is possible that the "Indian River, Fla." *J. zonalis* specimen is mislabeled and not actually from Florida. This is our current preferred hypothesis for the provenance of this specimen.

We have not been able to find any additional *J. zonalis* specimens with similar locality labels at USNM, (which now holds the Neumoegen collection) or at AMNH (which holds the Henry Edwards collection) (Leach 2013). The butterfly specimens that W. H. Edwards forwarded to Neumoegen from Wittfeld's "Indian River" collections were primarily duplicates (Calhoun 1994). If similarly labeled *J. zonalis* material can be found among the material in the W. H. Edwards collection (at the Carnegie Museum of Natural History (Leach 2013), which we have not yet had the opportunity to consult), it would lend additional support to the presence of *J. zonalis* in Florida during the 19$^{th}$ century.

## Concluding remarks

Based on all of the available lines of information, *J. zonalis* likely began to invade South Florida during the 1930s, establishing populations on the mainland by the 1940s. These populations may have been subject to extirpation, followed by reinvasion from either the Florida Keys or from Cuba, which appear to have far more stable populations, as has been suggested previously (Cech and Tudor 2005; Lalonde and Marcus 2019b). These invasion events of mainland Florida appear to be accompanied by increases in the

frequency of mitochondrial haplotype group A in both *J. zonalis* and *J. coenia* (Figure 3-2), suggesting that genetic exchange is taking place between these species during these invasion events. All of the *J. zonalis* (and likely *J. zonalis* hybrid) specimens attributed to Florida prior to the 1930s that we have examined have questionable provenance and without corroborating data should not be taken as evidence of earlier invasion events. The most likely source of the 1900s material *J. zonalis* attributed to Chokoloskee is Cuba, while the "Indian River" *J. zonalis* specimen was probably collected during the early 1880s but may not have been collected in Florida.

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## Connecting section: Chapter 3 to Chapter 4

In Chapter 3, I used museum collection data in combination with mitochondrial haplotype data to reconstruct the invasion history of *Junonia zonalis* in Florida. I also addressed anomalous species records from *Junonia* specimens from the early 1900's with collection data from Chokoloskee, Florida. Based on my analysis, it was possible to determine that the anomalous Chokoloskee collection records are associated with specimens that were most likely collected in Cuba.

Chapter 4 will address the hypothesis that the New World *Junonia* constitute a ring species. Originally the discontinuity in the *Junonia* ring was thought to occur in Cuba but this hypothesis was later falsified based on continuous gene flow in Cuba. A new apparent discontinuity was suggested for Panama and northern South America. This chapter will use a combination of life history, morphology, mitochondrial haplotype data, and biogeography to characterize gene flow within the genus and test the ring species hypothesis. It will also document the geographical distributions of each New World species and clarify how many species occur in the New World.

# Chapter 4: The Chronicles of *Junonia*: Testing the ring species hypothesis in the New World buckeye butterflies

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Author Contributions:

ML designed the study, did data collection, performed experiments, analyzed data and drafted the manuscript.

JM assisted with study design and statistical analysis, provided laboratory space, research materials, and revisions to the manuscript.

**Abstract**

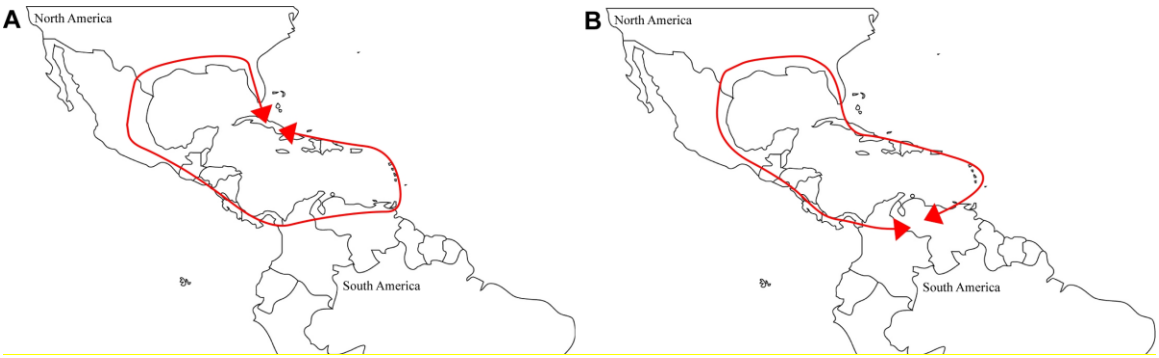
Ring species were long considered to illustrate a particular way in which species biodiversity can be generated by biogeographical processes, species divergence, and speciation through vicariance. The New World butterflies of the genus *Junonia* were proposed to constitute a ring species by Forbes (1928) with populations around the circumference of the Caribbean thought to be demonstrating continuity of gene flow, but with a discontinuity of gene flow and overlapping ends of the *Junonia* distribution found in Cuba. This original ring species hypothesis was later falsified by Gemmell and Marcus (2015) who showed that ongoing gene flow appeared to be taking place not only around the circumference of the Caribbean, but also among island populations, including Cuba. Based on limited sampling in Panama and northern South America, Gemmell and Marcus (2015) suggested that a possible discontinuity in gene flow may exist among *Junonia* populations in this area. I tested this hypothesis by extensively sampling and genotyping 17 *Junonia* species from Central and South America. Further, I characterized their morphological characteristics and estimated their biogeographic distributions and mitochondrial gene flow throughout this region. Based on these results, gene flow appears to be occurring between the species in Panama, northern South America, and throughout the New World, falsifying the hypothesis that *Junonia* is a ring species.

## Introduction

Ring species are of particular importance in biology as they can help scientists understand the processes of speciation, how organisms evolve through time, and how biodiversity is produced (Irwin *et al.* 2001; Blackmon and Demuth 2012; Martins *et al.* 2013). A ring species is a transitional state in the process of speciation by vicariance. Ring species occur where organisms within a taxon have a ring-shaped distribution around a region of unsuitable habitat. This distribution is characterized by genetic exchange as a function of distance at all points of the distribution with the exception of the overlapping ends of the ring where there is no gene flow between extreme forms (Cain 1954; Mayr 1963; Joseph *et al.* 2008; Blackmon and Demuth 2012; Gemmell and Marcus 2015). When a vicariance event interrupts gene flow around the ring, a single lineage splits into two distinct lineages (Mayr 1942). Although many potential ring species have been proposed, few have withstood scrutiny (Irwin *et al.* 2001), with the best example to date being the greenish warblers (Martins *et al.* 2013). The concept of ring species is a special case where taxonomic entities (regardless of taxonomic rank) within a ring are not reproductively isolated, so an entire genus might be encompassed within a single ring species. Within this framework, distinct species are defined exclusively by the patterns of reproductive isolation as they relate to biogeography (Kuchta and Wake 2016).

Forbes (1928) suggested that the *Junonia* butterflies of the New World form a ring species around the Caribbean, with the overlapping ends of the ring occurring in Cuba (Fig. 4-1 (A), (Forbes 1928)). This hypothesis was restated and popularized, but not tested by later authors (Mayr 1942; Mayr 1963; Remington 1985). Irwin et al. (2001) speculated that the New World *Junonia* were unlikely to be a ring species because of insufficient gene flow between circum-Caribbean populations. When the ring species hypothesis was tested using mitochondrial DNA markers, with one possible exception, there was evidence for ongoing gene flow throughout the ring, including between the *Junonia* species found in Cuba, the supposed region of range overlap. (Gemmell and Marcus 2015; Lalonde and Marcus 2019a). This falsified the ring species hypothesis as it had been originally formulated by Forbes (1928).

**Figure 4-1.** Representations of the ring species hypothesis for the genus *Junonia*. (A) represents the original ring species hypothesis proposed by Forbes (1928), where the ends of the ring overlap in Cuba. (B) represents the ring species hypothesis proposed by Gemmell & Marcus (2015) where the ends of the ring overlap in Northern South America after concluding that gene flow in Cuba and the Caribbean is continuous.




However, Gemmel and Marcus (2015) observed that there was an apparent major transition in mitochondrial haplotype frequencies at either the Isthmus of Panama or in northern South America (Fig. 4-1 (B)). To the north of Panama, haplotype group B is found almost exclusively (nearly 100% B) and to the south haplotype group A is prevalent (over 85% A in many populations) (Pfeiler *et al.* 2012; Gemmell and Marcus 2015; Lalonde and Marcus 2019b). From 1867 (when *Junonia vestina* was described) to 2008 it was thought that only four different *Junonia* species (*J. evarete, J. genoveva, J. vestina, J. coenia*) occurred in the New World (Brévignon 2004; Neild 2008). *Junonia evarete*, *J. genoveva*, and *J. vestina* were known to occur in South America, while *J. coenia* occurs primarily in North America. Twenty-first century taxonomic work suggests that there appears to be at least 18 New World *Junonia* species (with perhaps additional species that are yet to be described) with 15 known from Central and South America (Brévignon 2004; Neild 2008; Brévignon 2009; Brévignon and Brévignon 2012; Gemmell and Marcus 2015). Prior to the current study, the few already genotyped *Junonia* samples from Central and South America have been inadequate for understanding the diversity of *Junonia* and subsequently the genetic population structure found within this region. It has been unknown if an abrupt shift (coincident with a disruption of gene flow) in mitochondrial haplotypes occurs in Panama and the surrounding region, or if there is a long smooth transition

in the gene frequencies (no disruption of gene flow), similar to what has been observed in Cuba and the Caribbean (Gemmell and Marcus 2015). Additional sampling from Panama and the northern portion of South America should allow us to distinguish whether or not the genus *Junonia* is a conventional ring species based on the presence or absence of gene flow disruption in the region of overlap (Gemmell and Marcus 2015).

To further explain how mitochondrial haplotypes can be used to distinguish patterns of genetic exchange in *Junonia*, it should be noted that all species can be identified based on morphology, many have discrete ranges, and in geographic regions where genetic exchange is occurring, co-occurring *Junonia* species share very similar haplotype group frequencies. Thus, it is possible to measure haplotype group frequency differences between the species in the supposed area of ring species overlap/gene flow disruption described above. If gene flow is interrupted, we would expect *Junonia* forms on either side of the boundary to have different haplotype group frequencies, while any differences would be expected to be homogenized if substantial gene flow is taking place across the supposed boundary.

Allopatric speciation models focus on isolation by distance and speciation by vicariance at geographic barriers, which is considered to be one of the most effective ways to explain the divergence of species (Mayr 1942; Mayr 1963). Such barriers may include mountain ranges, deserts, bodies of water, or heavily forested areas depending on the biology of the specific organisms being considered. As many (but not all) flying organisms have long-range dispersal mechanisms, static geographic barriers to gene flow may not be an effective isolation mechanism. Allopatric speciation in such organisms, like some forms of North American *Junonia*, may have taken place in glacial refuges during glaciation maxima. After speciation, gene flow associated with secondary contact is currently thought to be taking place, as there is evidence that hybridization is occurring between *Junonia* species in the American Southwest and Mexico (Hafernik 1982; Lalonde and Marcus 2019b; Cong *et al.* 2020). If the genus *Junonia* is indeed a ring species, we would expect isolation by distance to be a principal driver of the divergence of haplotype groups and not allopatric speciation in isolated refuges.

Based on previous work done in *Junonia* there seems to be a gradient in the haplotypes with haplotype group A more prevalent in South America, haplotype B most prevalent in North and Central America, and with the Caribbean appearing to be an area of admixture between

populations carrying the two haplotype groups (Gemmell *et al.* 2014; Gemmell and Marcus 2015; Lalonde and Marcus 2019a; Lalonde and Marcus 2019b; Lalonde and Marcus 2020a). A careful examination of *Junonia* populations and species across the apparent transition zone in Panama and northern South America will identify the state of gene flow across the boundary and determine if there has been local adaption to extreme environmental conditions that is unshared with *Junonia* on the other side of the boundary. For example, there is some evidence that mitochondrial haplotype group B may be selectively favoured in more variable temperate climates, while haplotype group A maybe more favoured in tropical and subtropical habitats (Lalonde and Marcus 2019a).

In order to document gene flow occurring within the genus *Junonia* in the Western hemisphere and test if the genus displays gene flow characteristic of ring species, specimens from museum collections were consulted for 17 of the 18 confirmed New World *Junonia* species (Table 4-1). Excluded was *Junonia vestina*, a high elevation species that carries unique mitochondrial haplotypes and may be descended from a separate invasion of the New World by genus *Junonia* (Chapter 5; (Pfeiler *et al.* 2012; Gemmell and Marcus 2015; McCullagh 2016; Peters and Marcus 2017)). Since museum specimens often retain and preserve more mitochondrial DNA than nuclear DNA (Lalonde and Marcus 2020a; Lalonde and Marcus 2020b) and the experimental tools to readily distinguish mitochondrial haplotypes already exist, this was used to compare haplotypes and detect gene flow. If the genus *Junonia* displays a discontinuous pattern of haplotype frequencies across the proposed boundary, this evidence could be interpreted as being consistent with the ring species hypothesis (Fig. 1-2, Fig 4-1). On the other hand, if there is a continuous gradient of haplotype frequencies and a smooth transition across the proposed boundary, this would be inconsistent with the existence of a *Junonia* ring species (Fig. 1-3). In addition to testing the ring species hypothesis, documentation of the morphological and life history data for the 17 New World *Junonia* species was done in order to delimit species, as well as to characterize of geographic ranges for each species.

**Table 4-1**. The 18 *Junonia* species and associated subspecies in the Western Hemisphere. Classification of species and subspecies are based on morphological characteristics. An * indicates the 17 species that occur in the proposed ring species range.

| Species |
|---|
| *J. coenia* Hübner, 1822 * |
| *J. coenia bergi* Avinoff, 1926 |
| *J. coenia coenia* Hübner, 1822 |
| *J. divaricata* C. & R. Felder, 1867 * |
| *J. divaricata divaricata* C. & R. Felder, 1867 |
| *J. divaricata houlberti* Brévignon, 2008 |
| *J. divaricata mitraka* Brévignon, 2012 |
| *J. evarete* Cramer, 1779 * |
| *J. evarete dougueti* Brévignon, 2008 |
| *J. evarete evarete* Cramer, 1779 |
| *J. evarete occidentalis* C. & R. Felder, 1862 |
| *J. evarete pallens* C. & R. Felder, 1867 |
| *J. flirtea* (Fabricius, 1793) * |
| *J. fuscencens* (Butler, 1901) * |
| *J. genoveva* (Cramer, 1780) * |
| *J. genoveva constricta* C. & R. Felder, 1867 |
| *J. genoveva genoveva* (Cramer, 1780) |
| *J. genoveva vivida* Forbes, [1929] |
| *J. grisea* Austin & Emmel, 1998 * |
| *J. hilaris* C. & R. Felder, [1867] * |
| *J. infuscata* C. & R. Felder, 1867 * |
| *J. litoralis* Brévignon, 2009 * |
| *J. neildi* Brévignon, 2004 * |
| *J. neildi neildi* Brévignon, 2004 |
| *J. neildi varia* Grishin 2020 |
| *J. nigralis* Forbes [1929] * |
| *J. nigrosuffusa* Barnes & McDunnough, 1916 |
| *J. nigrosuffusa nigrosuffusa* Barnes & McDunnough, 1916 |
| *J. nigrosuffusa stemosa* Grishin 2020 |
| *J. oscura* Neild, 2008 * |
| *J. pacoma* Grishin 2020 * |
| *J. vestina* C. & R. Felder, 1867 |
| *J. vestina livia* Fruhstorfer, 1912 |
| *J. vestina vestina* C. & R. Felder, 1867 |
| *J. wahlbergi* Brévignon, 2008 * |
| *J. zonalis* C. & R. Felder, 1867 * |
| *J. zonalis michaelisi* Fruhstorfer, 1907 |
| *J. zonalis swifti* Brévignon, 2004 |
| *J. zonalis zonalis* C. & R. Felder, 1867 |

## Material and Methods

**Specimen Collection and Preparation.** A total of 2713 specimens from 17 *Junonia* species (Electronic Supplement IV, https://doi.org/10.34990/FK2/I6QUPN/CMDFSA) were selected based on geographic location (North America, South America, Central America, and Caribbean) from museum collections and private collectors. This included representatives of all described *Junonia* species from the New World except for *J. vestina.* Of the 2713 specimens, 917 were new samples from under-sampled regions of the lesser Antilles and from the core area of Central and South America (where the ends of the ring distribution are proposed to overlap) that had not been used in any previous analysis. Specimens were identified to species based on morphological characteristics (Electronic Supplement V, https://doi.org/10.34990/FK2/I6QUPN/FO5GVH) and assigned a laboratory code. DNA was extracted from a single butterfly leg per specimen with a Qiagen DNEasy Blood and Tissue Kit using either the assistance of a Qiagen QiAcube extraction robot (Qiagen, Düsseldorf, Germany) following the manufacturers using the animal tissue DNA program or manually as previously described (Gemmell and Marcus 2015) with several modifications to the standard Qiagen DNA extraction protocol to increase recovery of poorly preserved DNA from museum samples (Lalonde and Marcus 2019a; Lalonde and Marcus 2020b). After extraction, all DNA samples were stored at -20°C prior to genotypic analysis.

**Mitochondrial *Cytochrome c Oxidase I* (*COI*).** Diagnostic mitochondrial markers have already been established for determining haplotypes (Gemmell and Marcus 2015; Lalonde and Marcus 2019b; Lalonde and Marcus 2020b) and were applied in this study. Cytochrome *c* oxidase subunit I (*COI*) gene products were obtained using polymerase chain reaction (PCR) using gene specific primers. The first primer pair LCO1490 (GGT CAA CAA ATC ATA AAG ATA TTG G) and HCO2198 (TAA ACT TCA GGG TGA CCA AAA AAT CA) (Folmer *et al.* 1994) yielded the largest DNA fragment in this study, a 709 base pair product (including primer sequences, 658 bp without primers). PCR reaction conditions for LCO1490 and HCO2198 are as follows: 95˚C for 5 minutes; 35 cycles of 94˚C for 1 minute, 46˚C for 1 minute, 74˚C for 1 minute, and a final extension for 5 minutes at 72˚C; then a 4˚C hold. To visualize the PCR products obtained, a QIAxcel Advanced capillary electrophoresis instrument (Qiagen) fitted with a DNA Screening Cartridge with QX Size Markers (250 bp–4 kb v. 2.0) and QX Alignment Markers (50 bp-5 kb) using the AL320 electrophoresis method was used as previously reported

(Gemmell and Marcus 2015). Negative control amplifications (distilled deionized water instead of DNA extractions) were included for each PCR reaction to ensure that no contamination had occurred during the amplification process. If amplification in the negative controls was detected, all PCR reactions from that experiment were discarded and were repeated using fresh reagents. When satisfactory bands were obtained, a diagnostic triple restriction enzyme digest using AflIII, BseYI and BamHI restriction endonucleases (New England Biolabs (NEB), Ipswich, MA, USA) was performed to determine the haplotype of each specimen (Fig 2-1) as it is a rapid and cost-effective method of determining mitochondrial haplotypes for *Junonia* species (Gemmell and Marcus 2015; Lalonde 2016; Lalonde *et al.* 2018; Lalonde and Marcus 2019b; Lalonde and Marcus 2020b).

The diagnostic restriction enzyme digest was performed using 10 µL of each individual PCR product mixed with 2 µL NEB Buffer3, 2 µL BSA (10X, 1mg/mL), 4 µL deionized distilled water, 0.5 µL AflIII, 0.5 µL BseYI, and 1 µL BamHI, in a 1.5 mL microcentrifuge tube and incubated at 37˚C for 1 hour. Enzyme deactivation was done in a 70˚C water bath for 10 minutes, and separation of digest products was done using a QIAxcel Advanced instrument as described above. Haplotypes were assigned based on the size of the bands obtained from the restriction digest products: Haplotype Group A genotypes have a single BamHI cut site that produces 2 bands (419 bp and 290 bp), haplotype Group B cuts once with AflIII and BseYI which produces 3 bands (514 bp, 150 bp, 45 bp).

For PCR reactions where no products were obtained from the first amplification, reamplification was done using miniCOIF2 (ATA CTA TTG TTA CAG CCT CAT GC) (Gemmell *et al.* 2014) and HCO2198, that yields a shorter 569 base pair product (including primer sequences, 520 bp without primers). PCR conditions for miniCOIF2 and HCO2198 are as follows: 95˚C for 2 minutes; 5 cycles of 95°C for 1 minute, 46°C for 1 minute, 72°C for 30 seconds; 35 cycles of 95˚C for 1 minute, 53˚C for 1 minute, 72˚C for 1 minute; and a final extension for 5 minutes at 72˚C, then a 4˚C hold. Visualization of products were conducted in the same manner as described above. The PCR products were assigned to haplotype groups using the same diagnostic triple restriction enzyme digest as described above, as all of the enzyme cut sites are also present within this smaller fragment (Fig. 2-1, Table 2-2). The digested products were separated as before and haplotypes assigned by the size of the bands obtained: Haplotype Group

A with 2 bands (313 bp and 256 bp) due to the BamHI restriction site, and Haplotype Group B with 3 bands (351 bp, 218 bp, 68 bp) due to the AflII and BseYI cut sites.

If no PCR products were obtained from the miniCOIF2/ HCO2198 amplification, then they were reamplified using miniCOIF2 and miniCOIR3 (TAT TTC GAT CTG TTA AAA GTA TAG) (Gemmell and Marcus 2015) using the DNA from the miniCOIF2/HCO2198 amplification as the template. This reamplification yields a 501 base pair product (including primers, 454 bp without primers). This shorter amplification product does not include the BseYI restriction site, so digests of the PCR products did not include BseYI (rather 0.5 μL ddH2O was added to the digest instead). The digested products were visualized as described above, and haplotypes assigned based on band sizes obtained: Haplotype Group A produces 2 bands (256 bp and 245 bp) due to the BamHI restriction site, and Haplotype Group B produces 2 bands (351 bp and 150 bp) due to the AflIII cut site (Fig. 2-1, Table 2-2). The validations of this technique are presented in Chapter 2.

**Haplotype and species distribution maps.** The frequencies of haplotype A and B were pooled for all 2713 specimens first and plotted on a large-scale map of the Western Hemisphere by geographic location based on GPS coordinates to examine haplotype distributions. Maps were generated using R version 4.0.2 (R Development Core Team 2020) using ggmap (Kahle and Wickham 2013), ggplot2 (Wickham 2016), and dplyr (Wickham *et al.* 2018) packages. The haplotype proportions were represented on the maps using a colour gradient based on the proportion of haplotype group A, with red representing 100% haplotype group A (= 0% haplotype group B) and purple representing 0% haplotype group A (= 100% haplotype group B). The sample size for each geographic location is represented by the size of the circles. For individual species, only 2628 specimens were mapped (85 specimens were excluded for either being suspected hybrids or for incomplete locality data where only country of origin was available). The species in individual maps are represented by different shapes with the size of the symbol represented in the same way as described above. To create the Western Hemisphere map, the center point for longitude was set at 80 degrees West, latitude at 10 degrees North, zoom was set at 5 and scale at 2. Individual species maps were created to observe their distributions on a large scale using the Western Hemisphere map, employing the methods described above and are

included in Appendix I. To observe the species distributions and gradient of haplotypes, finer scale maps were created using the same methods described above but with adjustments to the center point, zoom and scale. Figure legends and axis labels were added to the maps using Canvas X Draw (Canvas GFX, Boston, Massachusetts).

**Testing for discontinuities in haplotype distributions.** In order to test for an apparent discontinuity in mitochondrial haplotype frequencies associated with high elevations in the Ecuadoran Northern Andes, Colombian Cordillera Oriental and Venezuelan Cordillera de Mérida mountain ranges (Blandin and Purser 2013), a line was plotted through Quito, Ecuador (0.1807 S,78.4678 W) and Barquisimeto, Venezuela (10.0678 N, 69.3474 W) to approximate the nearly straight, high elevation potential barrier to the movement of *Junonia* butterflies created by these mountain ranges. The first step in demonstrating the existence of a genetic discontinuity in along this line would be to reject the null hypothesis that there is a linear gradient of haplotype frequencies across this high elevation barrier. Thus, geographic locations were selected based on close geographic proximity to the line. Latitudinal coordinates are isometric, and one can approximate the shape of the Earth as a perfect sphere with exactly 111 km per degree latitude. Longitudinal coordinates are anisometric, meaning that the lines of longitude are farthest apart at the equator and converge at the poles, so one must correct for this distance as a function of latitude to place the projection on a Cartesian plane by converting the degrees of latitude to radians latitude so that the correct calculation of cosine can be performed.

Thus, the coordinates defining the boundary line and the GPS coordinates of sampling localities were then transferred from a spherical projection to a Cartesian projection by converting latitudinal coordinates by multiplying them by 111 km per degree and converting longitudinal coordinates by multiplying degrees of longitude by 111 km cos(Π degrees latitude/180) (NOAA 2021). On the Cartesian projection, the Quito-Barquisimeto boundary is defined by the equation (Eq. 1):

$$y = mx + c$$

where *y* is the transformed value (in km) for latitude, *x* is the transformed value (in km) for longitude, the slope *m* equals 1.005953154, and the intercept *c* equals 8741.676056. The straight-line distance *d* from each sampling location to the nearest point on the Quito-Barquisimeto boundary line was calculated using an equation derived algebraically by substituting Equation 1 into the Cartesian expression of the Pythagorean Theorem and solving for the length of the hypotenuse (Eq. 2):

$$d = \frac{(mx' - y' + c)}{\sqrt{(1 + m^2)}}$$

where *d* is the distance of the point from the line, *m* and *c* are defined as above, *x'* is the transformed value for longitude (in km) of each data point, and *y'* is the transformed value for latitude (in km) of each data point (Gore 2017). Negative values of *d* are indicative of sampling locations North and West of the Quito-Barquisimeto boundary line, while positive values of *d* are indicative of sampling locations South and East of the boundary line. Once *d* was calculated for each of the GPS coordinates (x-axis), they were plotted using Microsoft Excel (Redmond, Washington) with their associated proportion of haplotype group A (y-axis). The linear best-fit line was added to the plot and an $R^2$ value was obtained. The $R^2$ value was then converted to a t-statistic to obtain a p-value. The conversion and analysis described above were done using two different data sets: (1) all sample locations including locations where sample sizes were equal to 1 (117 locations total) and (2) only sample locations where sample sizes were four or greater (60 locations). Sample locations included all specimens in the data set from Belize, Bolivia, Brazil, Colombia, Ecuador, French Guiana, Guyana, Mexico, Panama, Peru, Suriname, Trinidad and Tobago, and Venezuela (Electronic Supplement IV, https://doi.org/10.34990/FK2/I6QUPN/CMDFSA).

To test for an apparent discontinuity in mitochondrial haplotype frequencies associated with the Caribbean, proposed by Forbes (1928) in Cuba, a linear boundary was plotted through Kingston, Jamaica (18.0179 N, 76.8099 W) and Guantanamo, Cuba (20.14 N, 75.2129 W). The

null hypothesis of no discontinuity predicts that there will be a linear gradient of haplotype frequencies across this line. Geographic locations were selected based on close geographic proximity to the line. This boundary line and the GPS coordinates of sampling localities were then transformed from a spherical projection to a Cartesian projection using the same methods described above for the Quito-Barquisimeto boundary line. On the Cartesian projection, the Kingston-Guantanamo line is defined by the equation Eq.1 with slope *m* equal to 0.873580779, and intercept *c* equal to 9082.794837. The straight-line distance *d* from each sampling location was calculated to the nearest point on the Kingston-Guantanamo line using Eq. 2 as described above. Once *d* was calculated for each of the GPS coordinates (x-axis), they were plotted using Microsoft Excel (Redmond, Washington) with their associated proportion of haplotype group A (y-axis). The linear best fit regression line was added to the plot and an $R^2$ value and p-value were obtained as described above. The conversion and analysis described above were done with a single data set only with locations with sample sizes of four or greater (87 locations) as it was demonstrated with the Quito-Barquisimeto dataset to have a more reliable signal and remove the noise associated with single specimen data points. Sample locations included all specimens in the data set from Bahamas, Cuba, Dominica, Dominican Republic, French Guiana, Guyana, Jamaica, Martinique, Puerto Rico, Trinidad and Tobago, Venezuela, and Florida, USA (Electronic Supplement IV https://doi.org/10.34990/FK2/I6QUPN/CMDFSA).

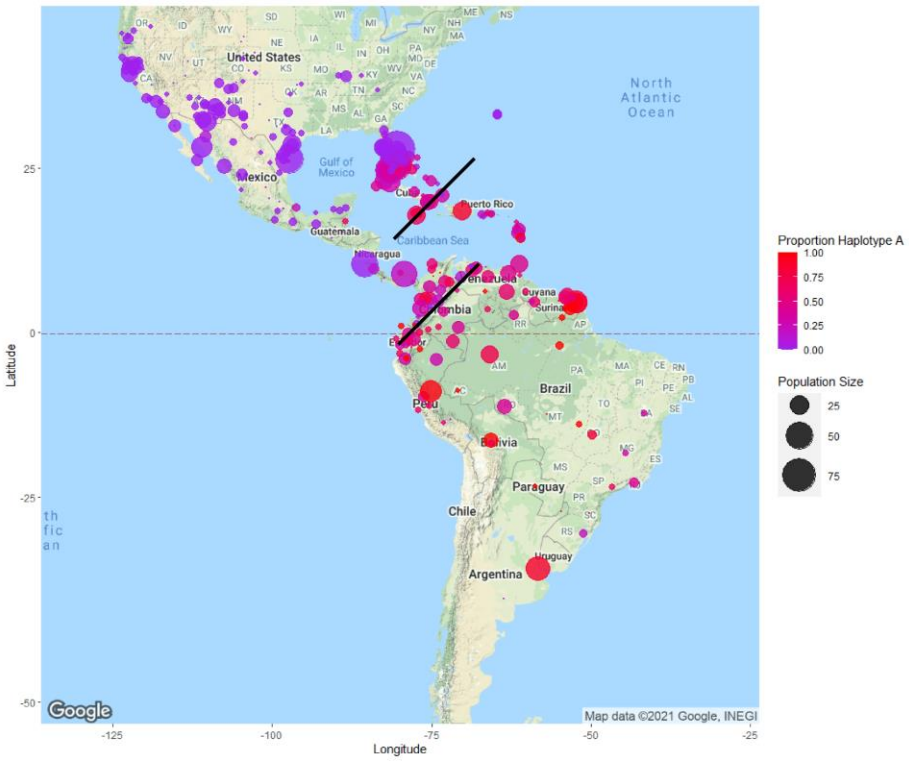
In order to determine if there may be an altitudinal difference in haplotype group frequencies associated with the Ecuadoran Northern Andes, Colombian Cordillera Oriental and Venezuelan Cordillera de Mérida mountain ranges, along our Quito-Barquisimeto line, all GPS coordinates that had a sample size of 4 or greater and determined altitude were used (Free Map Tools 2021). A graph of altitude (x-axis) was then compared to the proportion of haplotype A (y-axis) for each location using Microsoft Excel (Redmond, Washington). The linear best fit regression line was added to the plot and an $R^2$ value and a p-value were obtained as above.

## Results

***Junonia* species haplotype distributions.** The geographic and haplotype distributions of 18 *Junonia* species were compiled in the Western Hemisphere. Each is represented on a fine scale map (included in the main text below) and a second map that includes the entire Western Hemisphere (Appendix I). Most *Junonia* species include individuals carrying both haplotype A

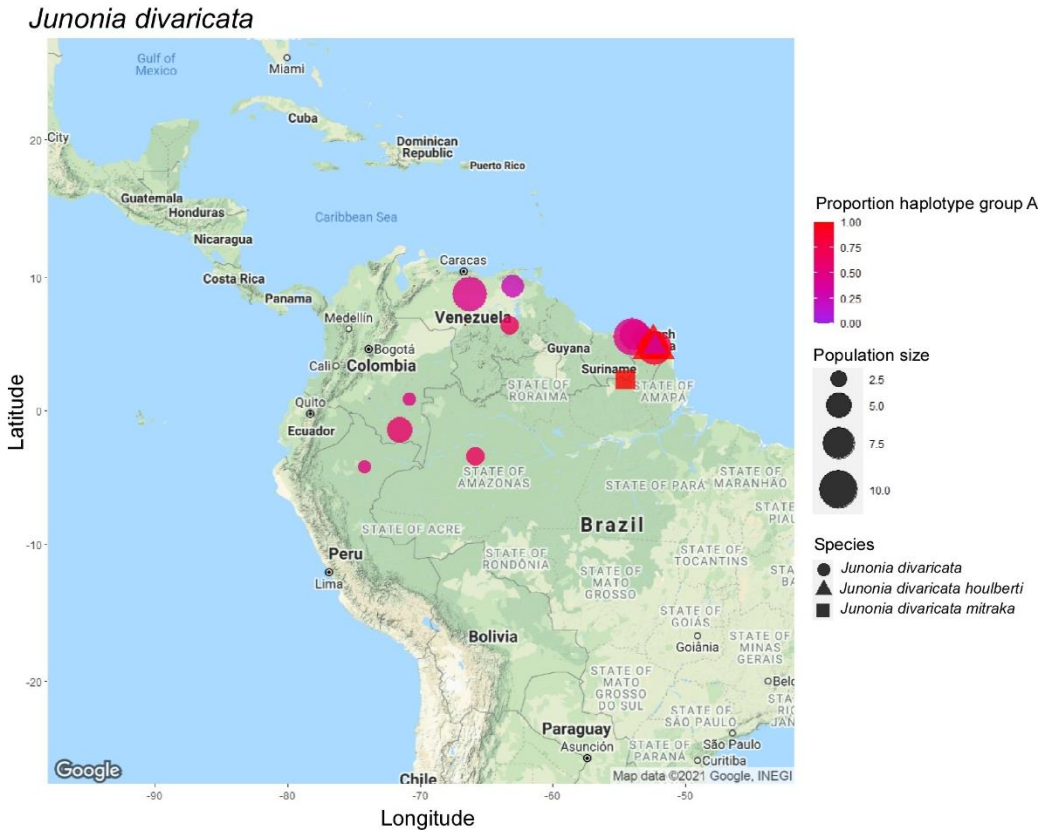
and B groups with the exception of *J. grisea*, which only carries alleles from haplotype group B. The distribution of haplotypes in the Western Hemisphere is displayed in Figure 4-2 that includes all 17 *Junonia* species in our dataset, composed of 2713 samples (2628 specimens and 85 specimens that were either suspected hybrid or had no locality data associated with it (i.e. only stated country of origin)). Like previous studies, the highest proportion of haplotype group A occurs in South America, the highest proportion of haplotype B is in North America , but the Caribbean seems to be a zone of genetic admixture with the highest proportion of B in the northern portions with a gradual increase in A in the more southern portions. Based on all species in our data set (Fig. 4-2), there appear to be two transition zones: (1) Between Central America and northern South America and (2) between Florida, USA, Cuba, and the northern Caribbean. These two transition zones are consistent with previously proposed transition zones (Forbes 1928; Gemmell and Marcus 2015) discussed above.

**Figure 4-2.** Proportion haplotype group A in the Western Hemisphere for all 2713 specimens. Proportion of haplotype group A is represented using a colour gradient with red representing 100% group A and purple representing 0% group A. The population size is represented by the size of the circles on the map. The hypothesized Kingston-Guantanamo (Caribbean) and Quito-Barquisimeto (South America) lines associated with possible genetic biogeographic discontinuities are also indicated on this map.




For *J. divaricata*, I obtained a total of 86 specimens with 69 from the nominate form (*J. divaricate divaricata*), as well as 14 *J. divaricata houlberti* and three *J. divaricata mitaraka* (Fig. 4-3). Haplotype group A is most predominant haplotype group found for this species, although group B is also found. *Junonia d. divaricata*'s range includes Northern Brazil, western Colombia, French Guiana, Northeastern Peru, and Venezuela. Haplotype group A was found to be the predominate haplotype group, but group B also occurs in this species. *Junonia d. houlberti* and *J. d. mitaraka* were found to be restricted to localities in French Guiana but with the similar haplotype group frequencies as found in the nominate form.

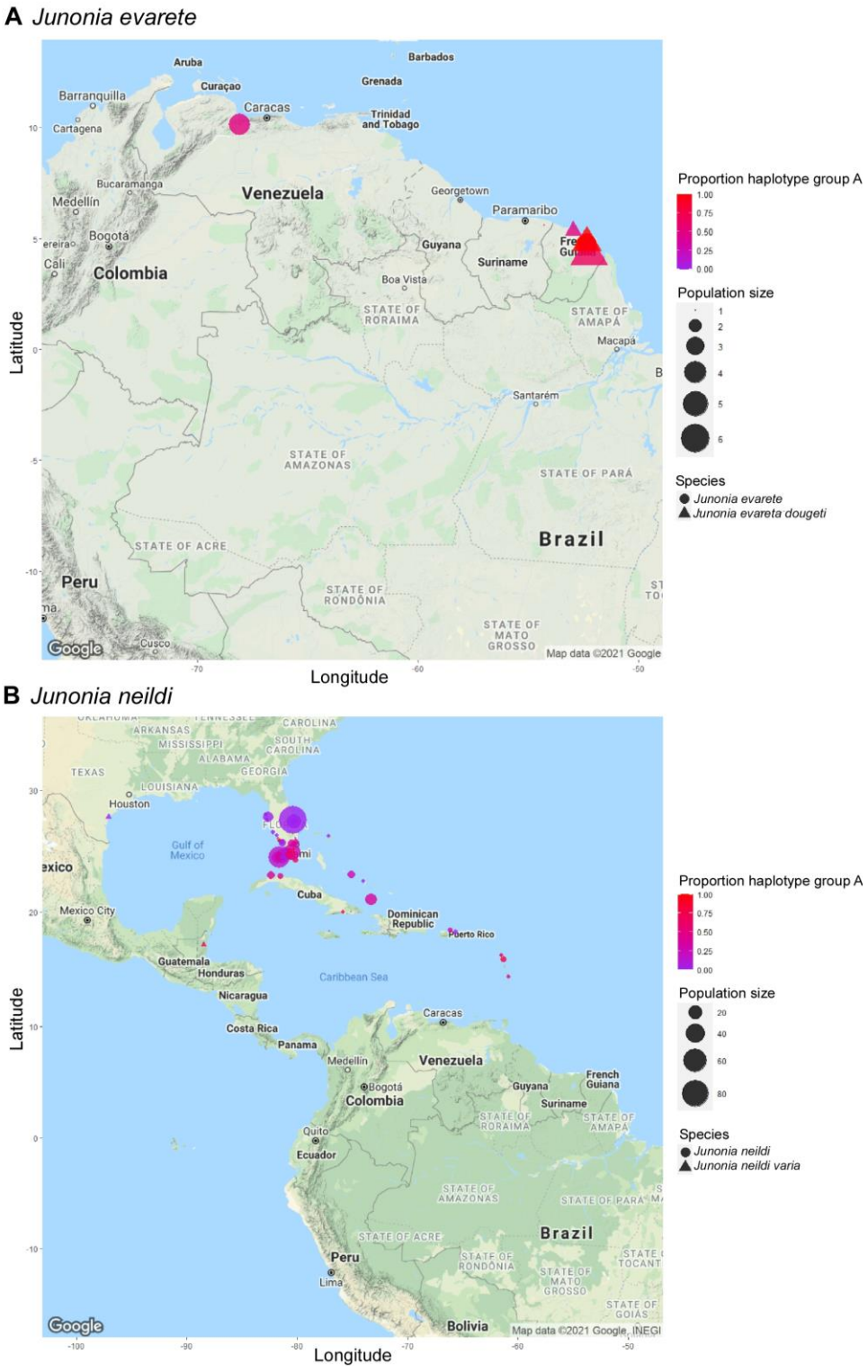
**Figure 4-3.** Species distribution and proportion haplotype A for *J. divaricata* (86 samples). Proportion of haplotype group A is represented using a colour gradient with red representing 100% group A and purple representing 0% group A. Subspecies designations are represented using different shapes and the population size is represented by the size of the shapes on the map.




We obtained 27 specimens for *Junonia evarete,* 14 from the nominate form, *J. evarete evarete,* and 13 for *J. evarete dougeti* (Fig. 4-4 (A)). The range of *J. e. evarete* includes northeastern Brazil, French Guiana, Suriname, and northern Venezuela. The predominant haplotype group was found to be haplotype group A, but group B also occurs in this species. *Junonia e. dougeti* were found to be restricted localities in French Guiana but with the same pattern or haplotype groups as the nominate subspecies. For *Junonia neildi,* I obtained a total of 444 samples, 438 from the nominate form *J. neildi neildi* and six samples for *J. neildi varia* (Fig. 4-4 (B)). The nominate form, *J. n. neildi*'s range includes the Bahamas, Cuba, Guadeloupe, Jamaica, Martinique, the east coast of Mexico, Puerto Rico, and Florida, USA. The predominate haplotype group was haplotype group B but group A also occurred in this species with higher frequency in the southern portion of the range. *Junonia n. varia* were found from Belize and Texas, USA, with the same pattern of haplotype group frequencies as the nominate subspecies.

**Figure 4-4.** Species distribution and proportion haplotype A for *Junonia evarete* (A) (27 samples) and *J. neildi* (B) (444 samples). Proportion of haplotype group A is represented using a

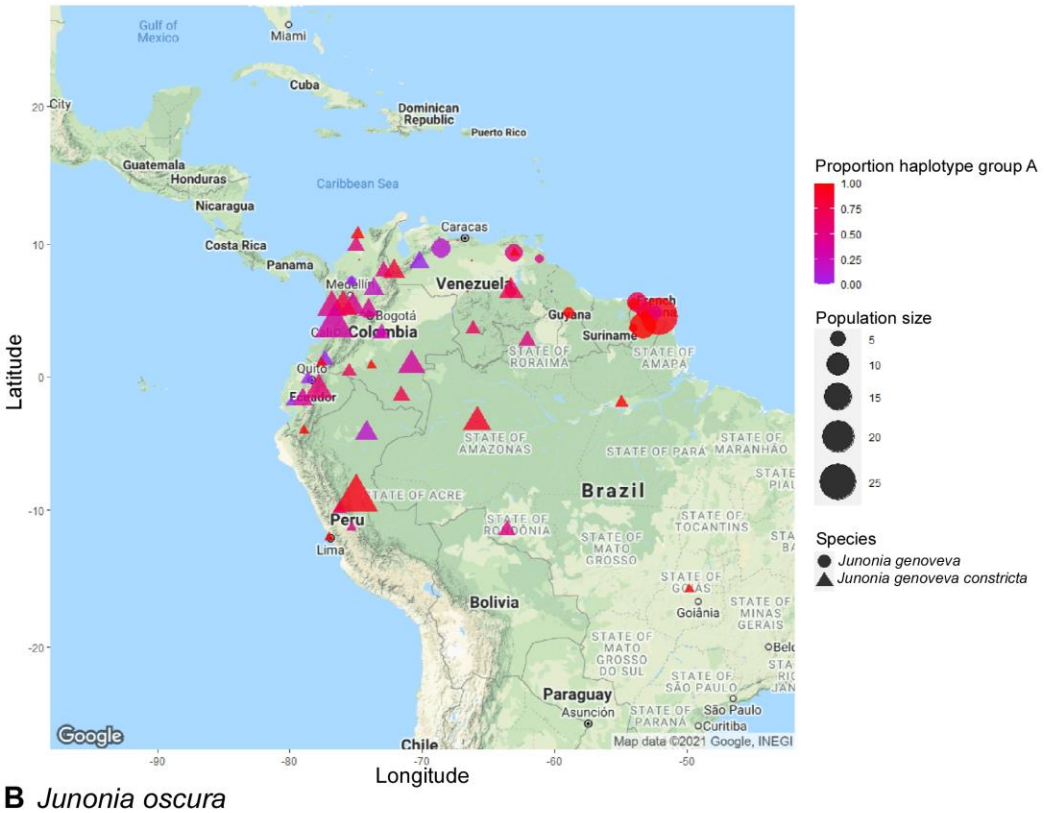
colour gradient with red representing 100% group A and purple representing 0% group A. Species designations are represented using different shapes and the population size is represented by the size of the shapes on the map.



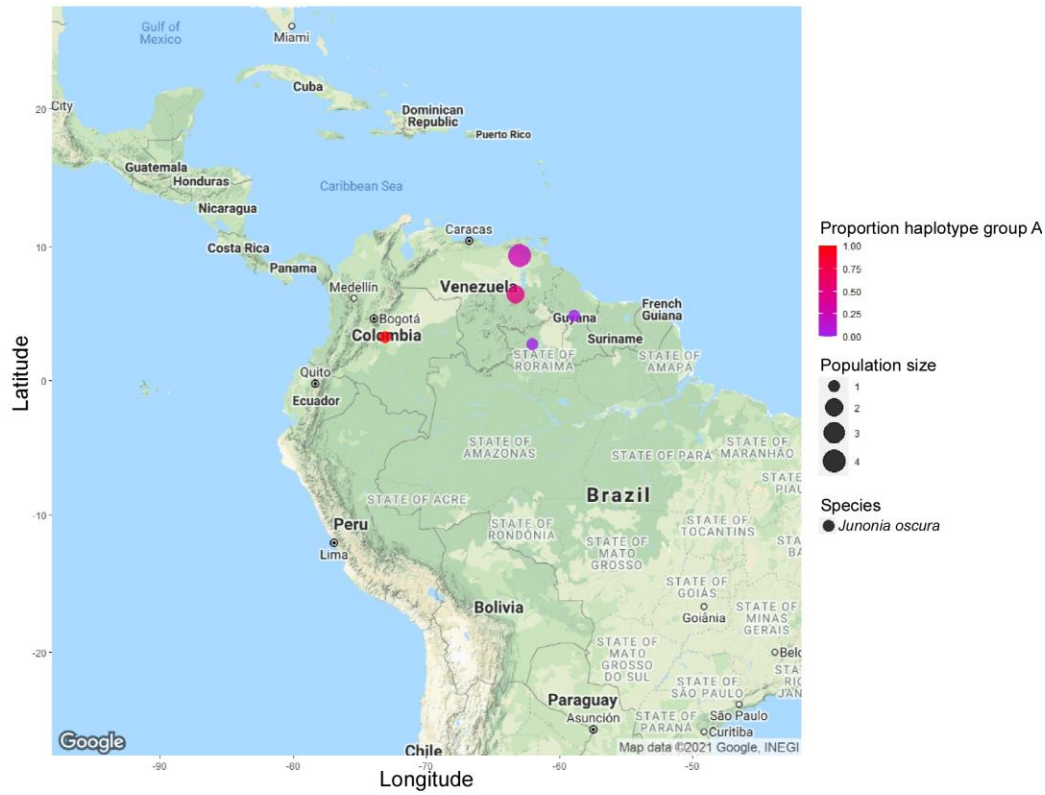
A total of 317 specimens for *Junonia genoveva* were obtained, 108 from the nominate form, *J. genoveva genoveva* and 209 *J. genoveva constricta* (Fig. 4-5 (A)). *Junonia g. genoveva*'s range includes northern Brazil, Colombia, French Guiana, Guyana, Suriname, and Venezuela. The predominate haplotype group was haplotype group A, but group B also occurs in this species although at lower frequency. *Junonia g. constricta* were found in western locations in Brazil, Colombia, Ecuador, Panama, Peru, and Venezuela with the same pattern or haplotype groups as the nominate form, except for a higher frequency of haplotype group A in the northern parts of South America. *Junonia oscura* has no subspecies described and I was able to obtain samples from nine specimens (Fig. 4-5 (B)). This species range includes Colombia, Venezuela, Guyana, and northern Brazil. The predominate haplotype group was haplotype group A but group B also occurs in this species with higher frequency in the southern portion of the range.

**Figure 4-5.** Species distribution and proportion haplotype A for *Junonia genoveva* (A) (317 samples) and *J. oscura* (B) (9 samples). Proportion of haplotype group A is represented using a colour gradient with red representing 100% group A and purple representing 0% group A. Species designations are represented using different shapes and the population size is represented by the size of the shapes on the map.

**A** *Junonia genoveva*



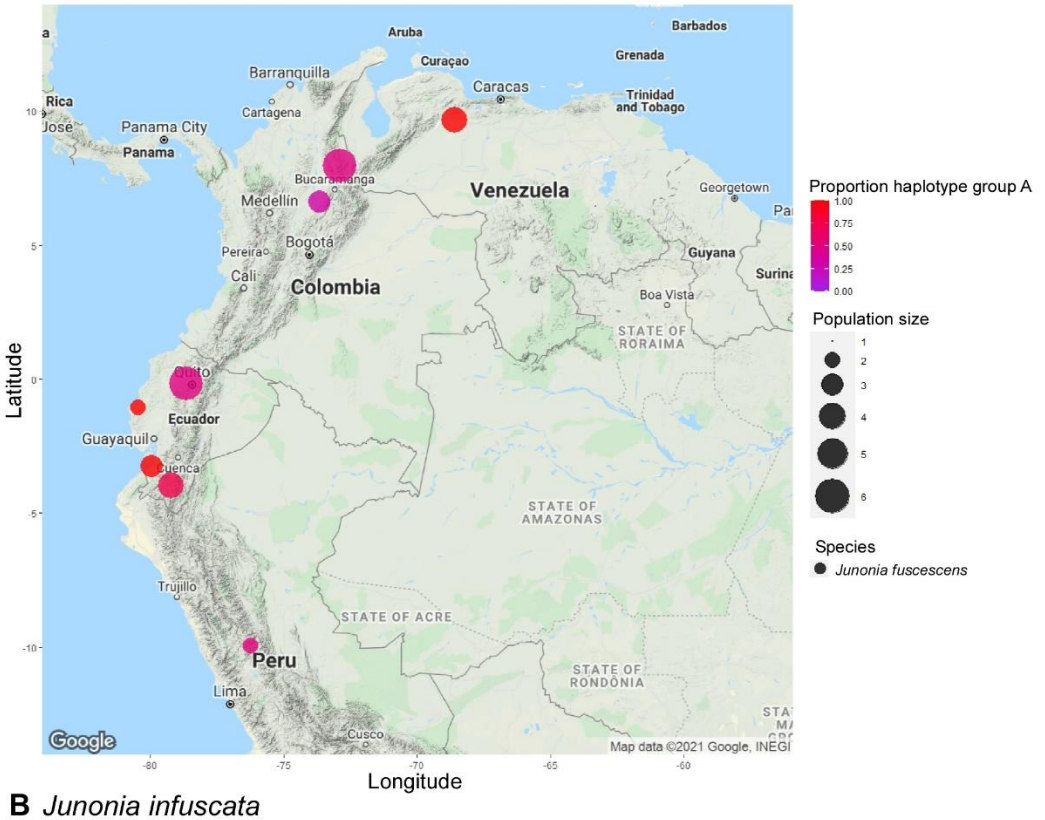

**B** *Junonia oscura*



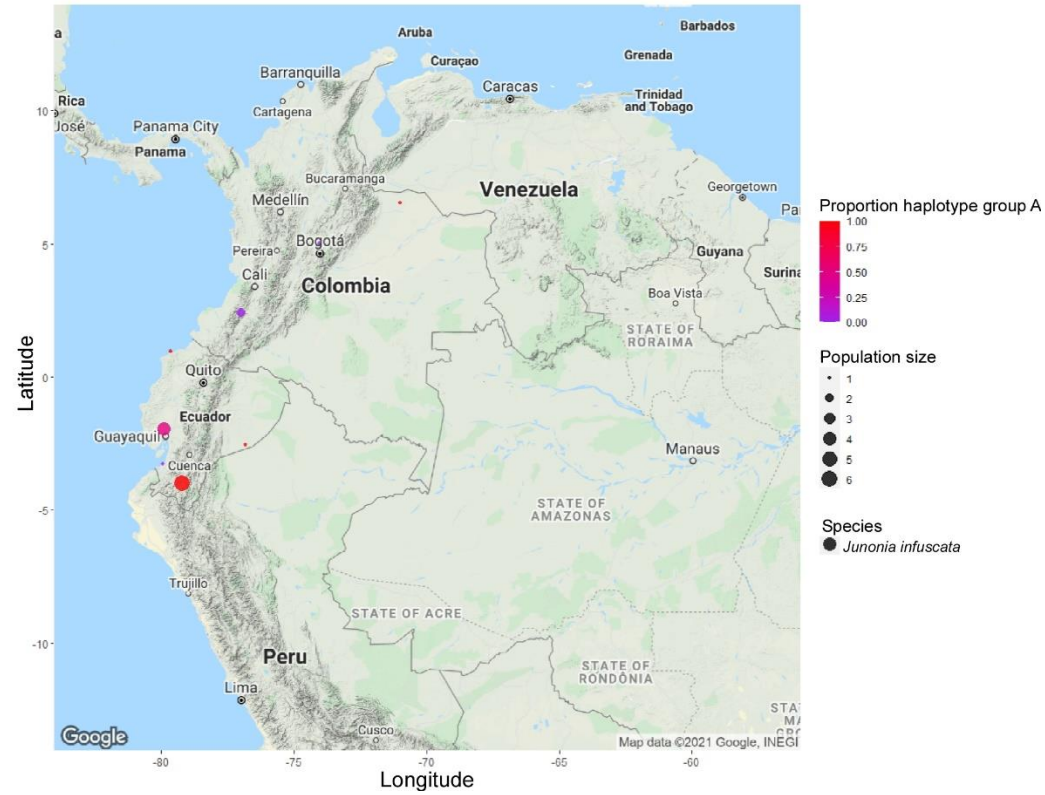
A total of 34 specimens for *Junonia fuscescens* were obtained for this study (Fig. 4-6 (A)). The range of *J. fuscescens* includes Colombia, Ecuador, Peru, and Venezuela. With the exception of Venezuela, where some specimens were collected to the east of the mountains, all specimens were collected to the either the north or west of the Ecuadorian Northern Andes, Colombian Cordillera Oriental, and Venezuelan Cordillera de Mérida mountain ranges. The predominate haplotype group was haplotype group A, but group B also occurs in this species at low frequency. I obtained a total of 17 samples for *Junonia infuscata*, and this species range includes Colombia, and Ecuador (Fig. 4-6 (B)). The predominant haplotype group was haplotype group A in the southern portion of the range and group B in with higher frequency in the northern portion of the range.

**Figure 4-6.** Species distribution and proportion haplotype A for *Junonia fuscescens* (A) (34 samples) and, *J. infuscata* (B) (17 samples). Proportion of haplotype group A is represented using a colour gradient with red representing 100% group A and purple representing 0% group A. Species designations are represented using different shapes and the population size is represented by the size of the shapes on the map.

**A** *Junonia fuscescens*



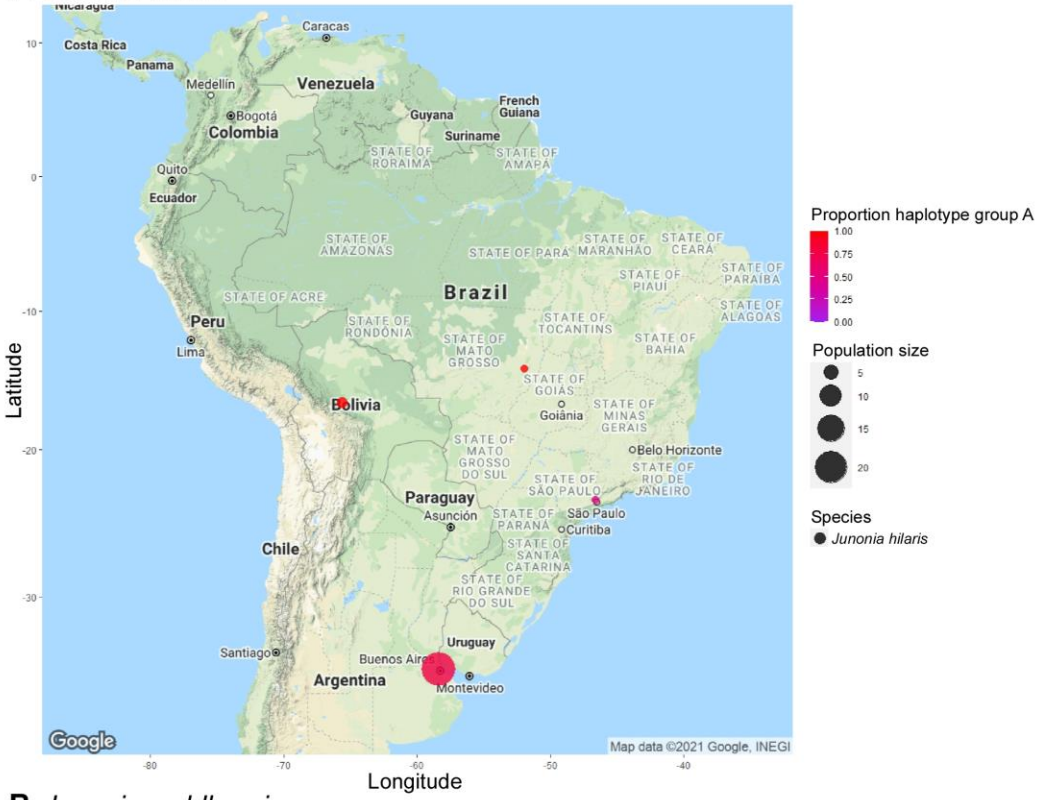

**B** *Junonia infuscata*



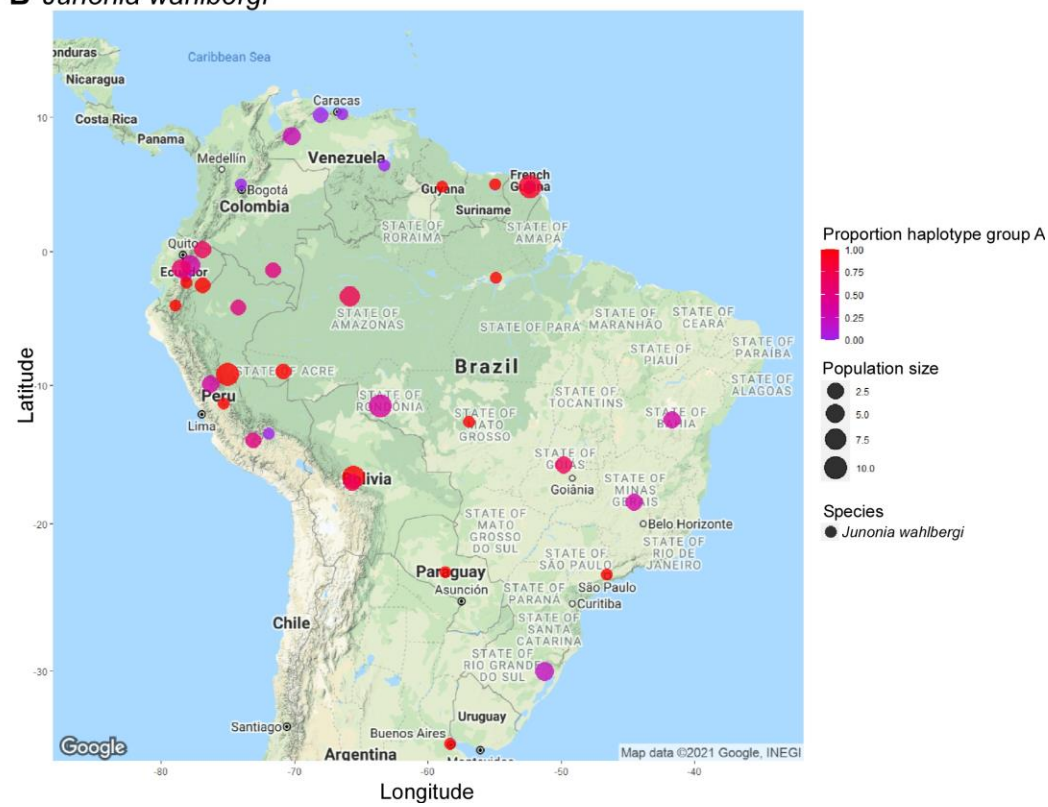
A total of 31 specimens for *Junonia hilaris* were obtained for this study (Fig. 4-7 (A)). The range of *J. hilaris* includes the some of the most southern portions of our study region including Bolivia, southern portions of Brazil and Argentina. The predominant haplotype group was haplotype group A, but group B also occurred in this species at low frequency. For *Junonia wahlbergi*, a total of 116 samples were genotyped (Fig. 4-7 (B)). This species is found throughout South America including Argentina, Bolivia, Brazil, Colombia, Ecuador, French Guiana, Paraguay, Peru, Suriname, and Venezuela. Without exception all specimens were collected to the either the south or east of the Ecuadoran Northern Andes, Colombian Cordillera Oriental, and Venezuelan Cordillera de Mérida mountain ranges. Haplotype group A predominated in this species with some exceptions in the northern parts of the range where group B occurred.

**Figure 4-7.** Species distribution and proportion haplotype A for *Junonia hilaris* (A) (31 samples) and, *J. wahlbergi* (B) (116 samples). Proportion of haplotype group A is represented using a colour gradient with red representing 100% group A and purple representing 0% group A. Species designations are represented using different shapes and the population size is represented by the size of the shapes on the map.

**A** *Junonia hilaris*



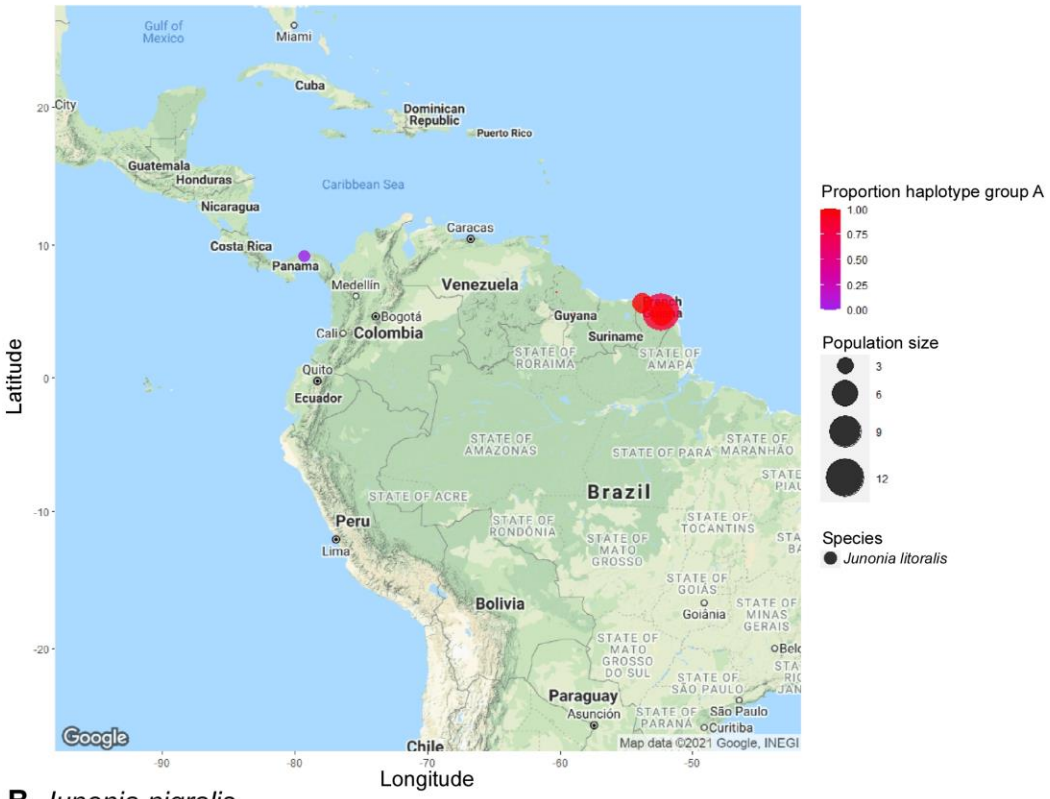

**B** *Junonia wahlbergi*



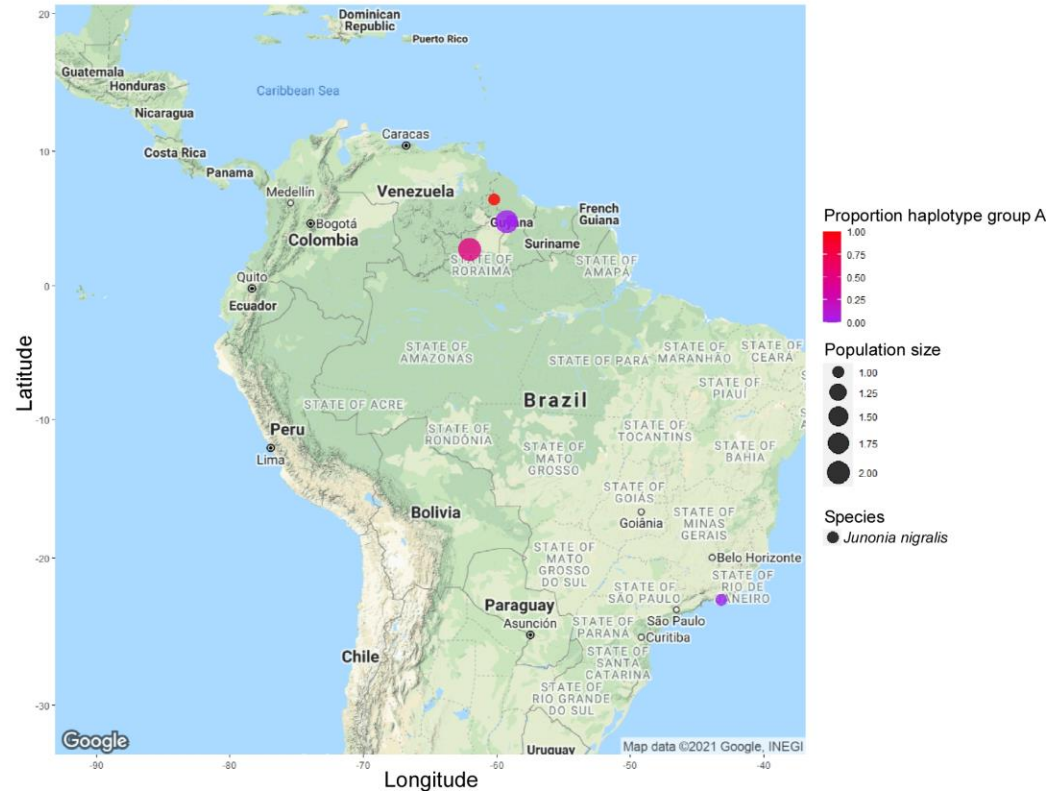
For the mangrove feeding species, *Junonia litoralis* 27 specimens were obtained (Fig. 4-8 (A)). This species was found in coastal regions from Costa Rica, Panama, Guyana, and French Guiana. The predominate haplotype group  in Guyana and French Guiana was haplotype group A, but group B was predominant in Costa Rica and Panama. Samples for *Junonia nigralis* were scarce with only seven samples in the entire data set (Fig. 4-8 (B)). Specimens obtained and sampled were from Northern Brazil, Rio De Janeiro, Brazil, and Guyana. In each country the northern parts had a high proportion of haplotype A while the southern portions had a high proportion of haplotype group B.

**Figure 4-8.** Species distribution and proportion haplotype A for *Junonia litoralis* (A) (27 samples) and, *J. nigralis* (B) (7 samples). Proportion of haplotype group A is represented using a colour gradient with red representing 100% group A and purple representing 0% group A. Species designations are represented using different shapes and the population size is represented by the size of the shapes on the map.

**A** *Junonia litoralis*

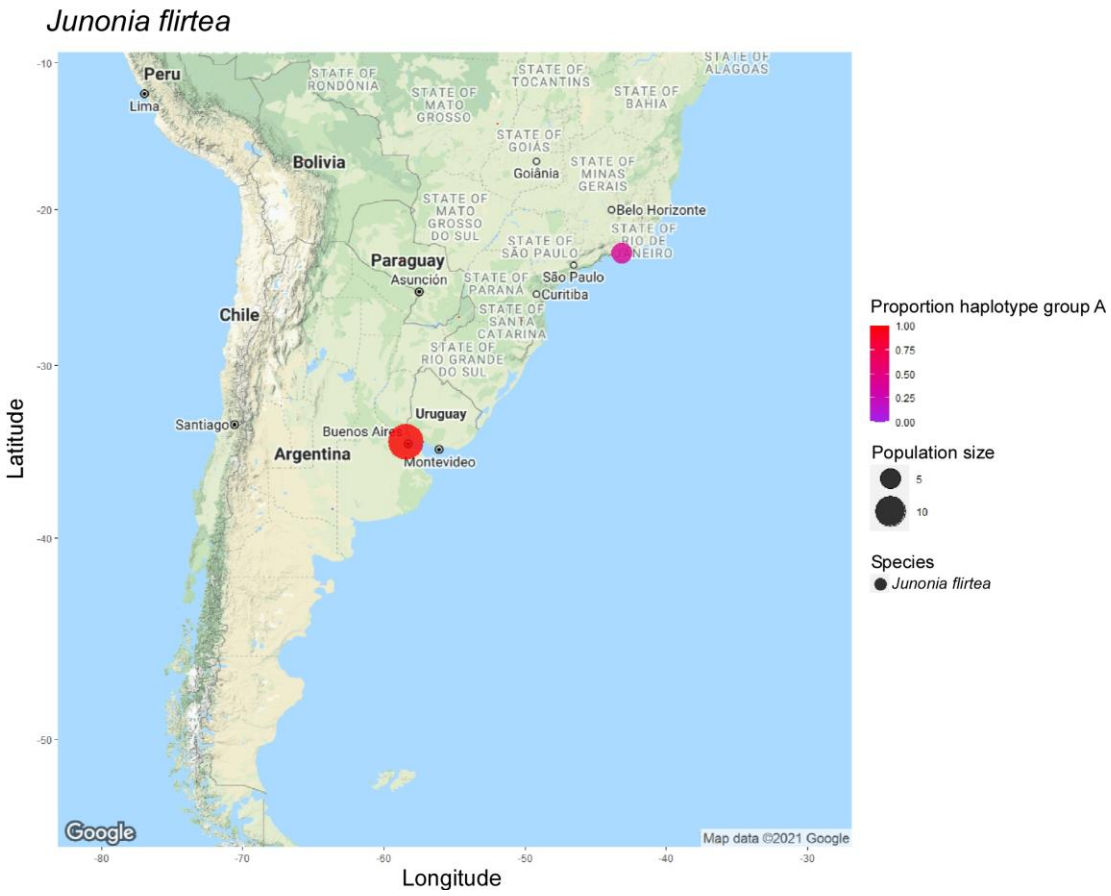



**B** *Junonia nigralis*



Twenty-five specimens of *Junonia flirtea* were obtained for this study (Fig. 4-9 (A)). The range of *J. flirtea* based on our sampling includes Argentina, Bolivia, Brazil, and Paraguay, the most southern portions of our study area. The predominant haplotype group for this species was group A with very few samples having haplotype group B alleles in the northern portions of the range, specifically in Brazil.

**Figure 4-9.** Species distribution and proportion haplotype A for *Junonia flirtea* (25 samples). Proportion of haplotype group A is represented using a colour gradient with red representing 100% group A and purple representing 0% group A. Species designations are represented using different shapes and the population size is represented by the size of the shapes on the map.

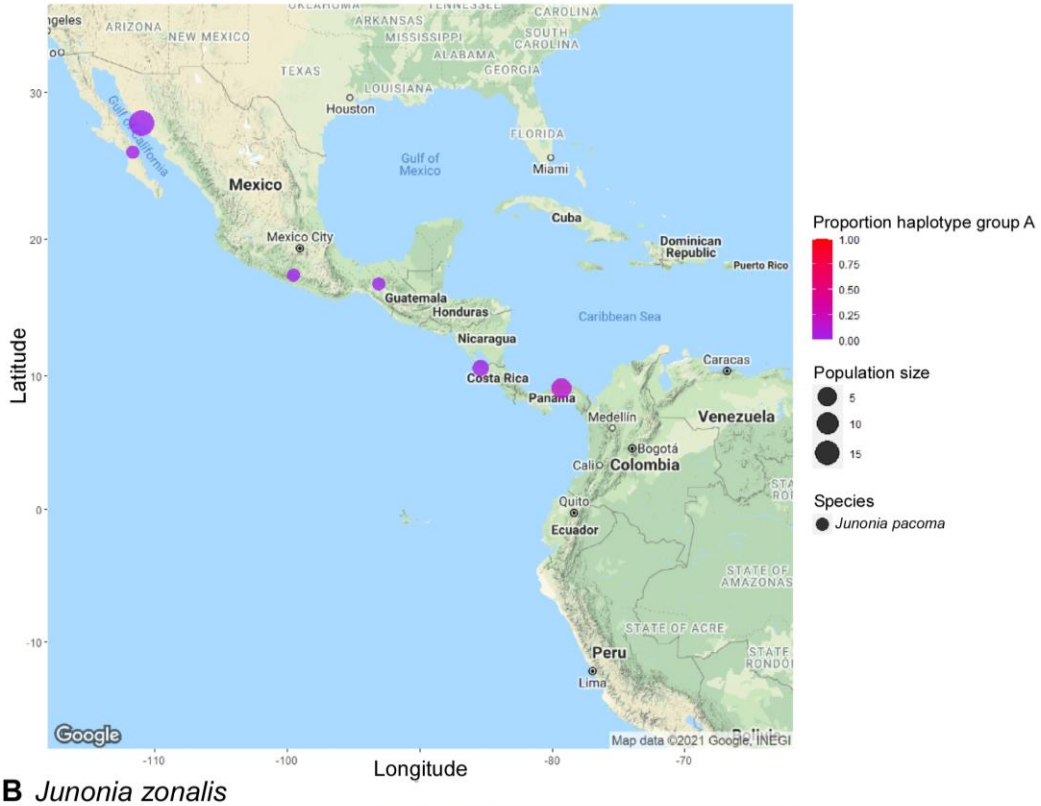



Twenty-eight *Junonia pacoma* samples were available for our study (Fig. 4-10 (A)). *Junonia pacoma* occurred in Mexico, Costa Rica, and Panama. The predominant haplotype

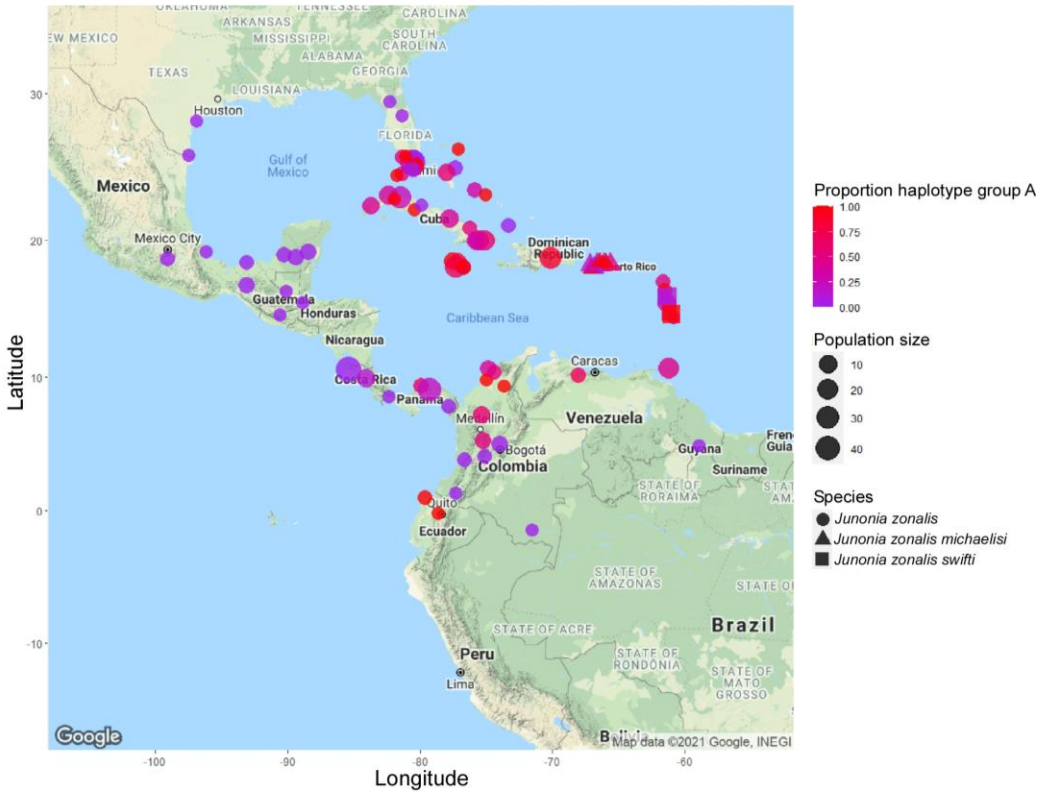
group was haplotype group B, but group A also occurred at low frequency in the southern range of this species. For *Junonia zonalis*, I obtained a total of 498 samples, 459 from the nominate form *J. zonalis zonalis*, 21 *J. zonalis michaelisi* and, 18 *J. zonalis swifti* (Fig. 4-10 (B)). *J. z. zonalis*'s range is the largest in our dataset and includes Antigua, the Bahamas, Colombia, Costa Rica, Cuba, Dominica, Dominican Republic, Guadeloupe, Guatemala, Guyana, Jamaica, Martinique, Mexico, Panama, Trinidad and Tobago, Venezuela, and in the United States, in Florida and Texas. The predominant haplotype group in the Western portion of the range is group B and in the Eastern portion of the range group A. The vast majority of *J. zonalis* occur to the northwest of the Ecuadoran Northern Andes, Colombian Cordillera Oriental, and Venezuelan Cordillera de Mérida mountain ranges, with the exception of a single sample from Guyana, a single state in Colombia (Amazonas), Venezuela, and the southern Caribbean island populations. *Junonia z. michaelisi* is found solely in Puerto Rico and *J. z. swifti* only from the Lesser Antilles (represented here by Guadeloupe and Martinique). The two subspecies show consistent haplotype distributions as many other *Junonia* species, with the highest proportion of haplotype group B in the northern parts of their ranges and haplotype group A in the southern parts.

**Figure 4-10.** Species distribution and proportion haplotype A for *Junonia pacoma* (A) (28 samples) and, *J. zonalis* (B) (498 samples). Proportion of haplotype group A is represented using a colour gradient with red representing 100% group A and purple representing 0% group A. Species designations are represented using different shapes and the population size is represented by the size of the shapes on the map.

**A** *Junonia pacoma*



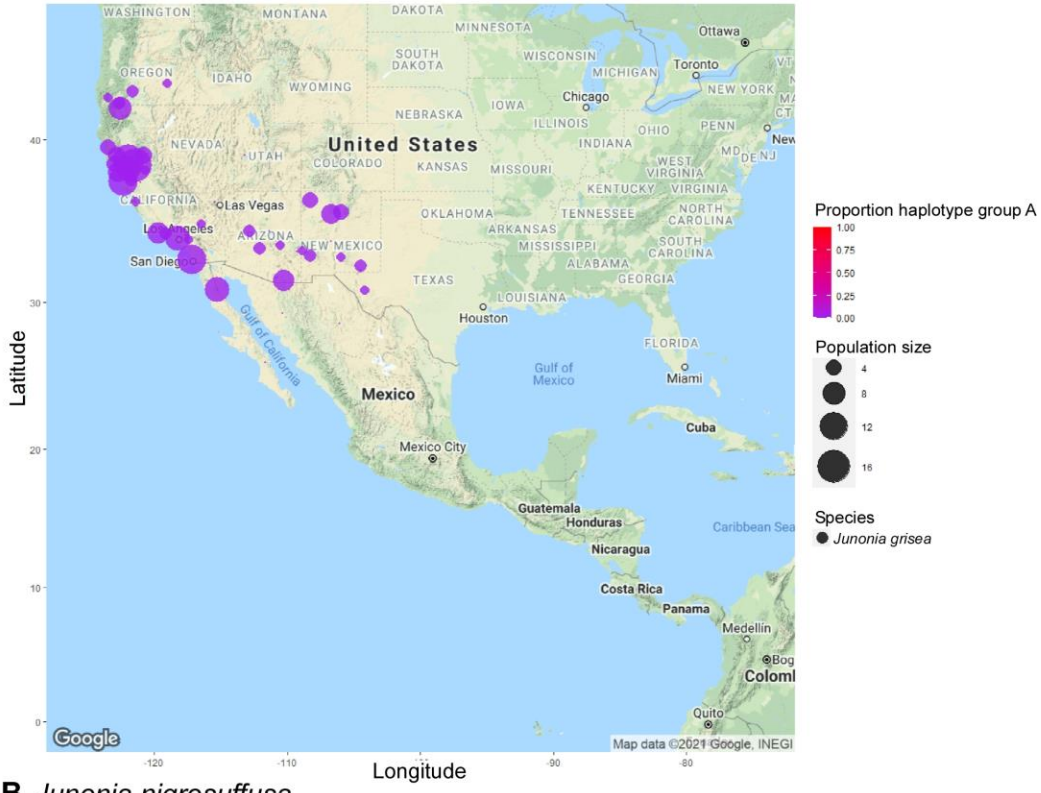

**B** *Junonia zonalis*



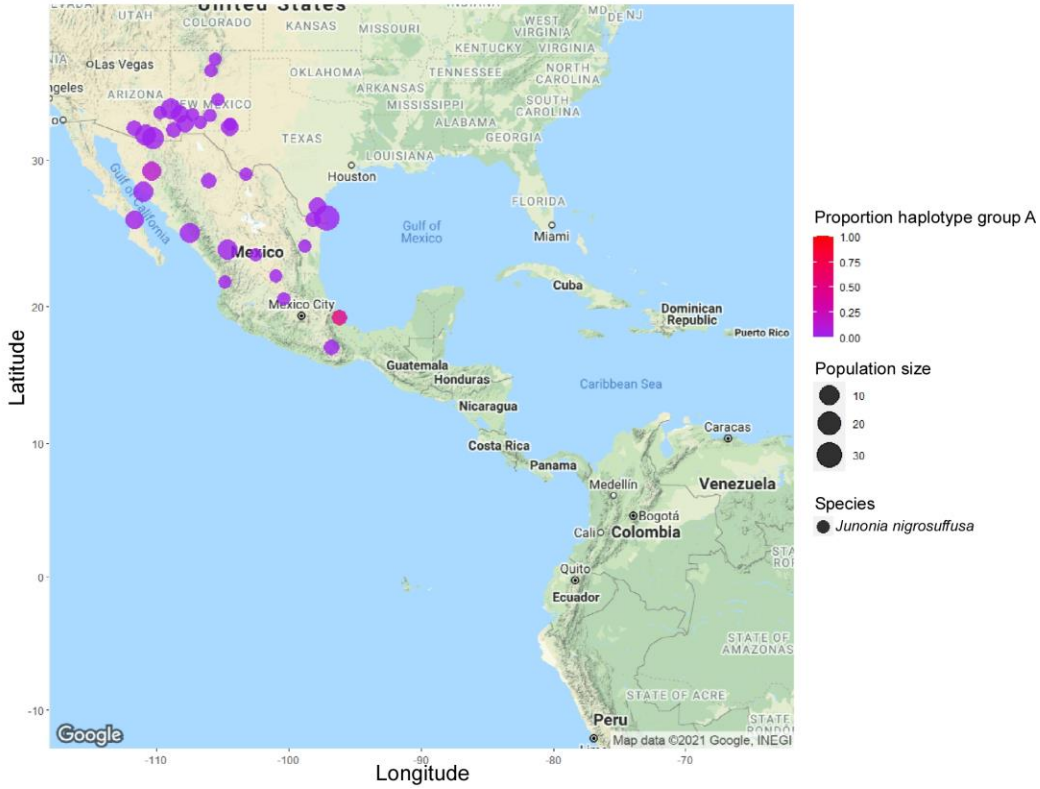
A total of 218 specimens of *Junonia grisea* were obtained for this study (Fig. 4-11 (A)). *Junonia grisea* is restricted to locations in the western United States and Mexico based on our sampling which is consistent with previous studies (Lalonde and Marcus 2019b), including Arizona, California, Nevada, New Mexico, Oregon, Texas, Utah in the United States, and the northern parts of Mexico. The haplotype distribution for this species is exclusively haplotype group B. For *J. nigrosuffusa*, I obtained a total of 157 samples were obtained for analysis (Fig. 4-11 (B)). As with *J. grisea*, *J. nigrosuffusa* is restricted to the similar locations in the western United States and Mexico which is consistent with previous studies (Lalonde and Marcus 2019b). This includes Arizona, New Mexico, and Texas in the United States and it was found throughout Mexico. The haplotype distribution for this species is almost exclusively haplotype group B, with some exceptions in Southern and Eastern Mexico.

**Figure 4-11.** Species distribution and proportion haplotype A for *Junonia grisea* (A) (218 samples) and, *J. nigrosuffusa* (B) (157 samples). Proportion of haplotype group A is represented using a colour gradient with red representing 100% group A and purple representing 0% group A. Species designations are represented using different shapes and the population size is represented by the size of the shapes on the map.

**A** *Junonia grisea*

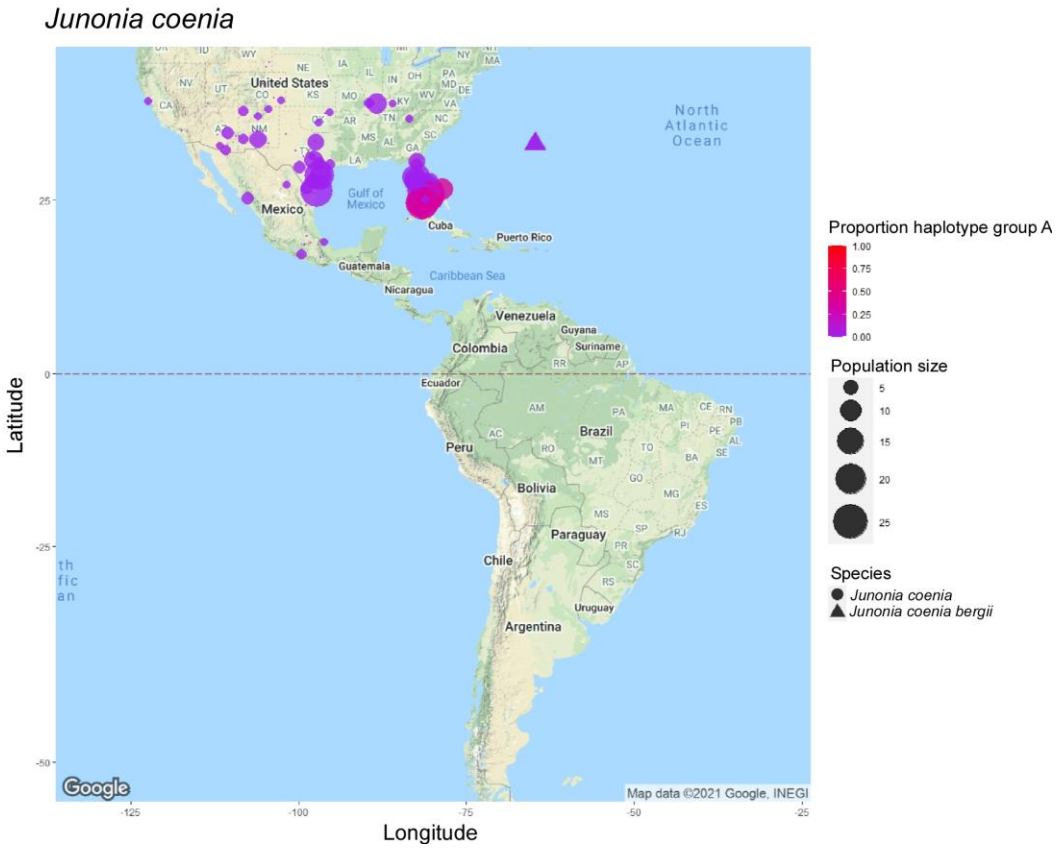



**B** *Junonia nigrosuffusa*



A total of 587 *Junonia coenia* specimens were obtained for this study (Fig. 4-12). The range of the nominate form, *J. coenia coenia*, is consistent with previous studies (Lalonde *et al.* 2018; Lalonde and Marcus 2019b), occurring only in the northern portions of our study area which includes the Bahamas, Cuba, Mexico, and in the United States, Arizona, California, Colorado, Florida, Kentucky, Missouri, New Mexico, North Carolina, Oklahoma, Texas, Utah and Wyoming. The predominant haplotype group for the nominate form was almost exclusively haplotype B except for Florida, USA, Cuba, and the Bahamas where haplotype group A occurs with some frequency. The subspecies, *J. coenia bergii*, is restricted to Bermuda and only carries haplotype group B which is consistent with previous studies (Peters and Marcus 2017).

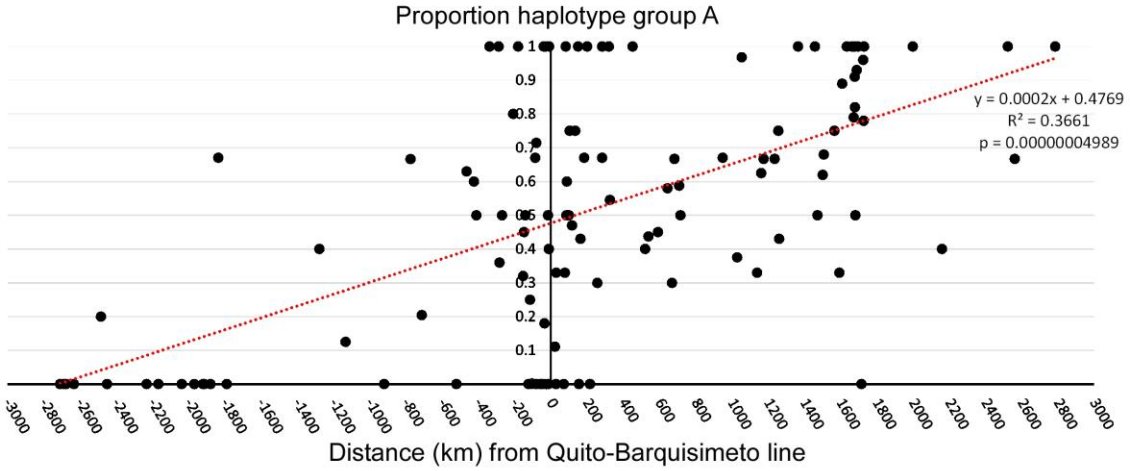
**Figure 4-12.** Species distribution and proportion haplotype A for *Junonia coenia* (587 samples). Proportion of haplotype group A is represented using a colour gradient with red representing 100% group A and purple representing 0% group A. Species designations are represented using different shapes and the population size is represented by the size of the shapes on the map.



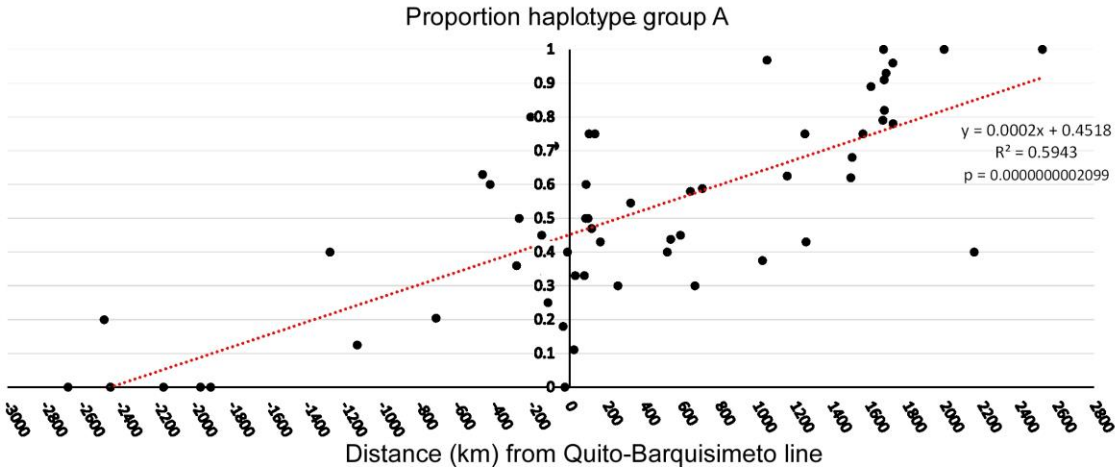
**Discontinuities in haplotype distributions.** Based on the haplotype distribution data (Fig. 4-2) across all species and the predictions of the ring species hypothesis, efforts were made to detect a discontinuity in haplotype distributions for the genus *Junonia*. The first possible discontinuity appeared to be associated with the Ecuadoran Northern Andes, Colombian Cordillera Oriental and Venezuelan Cordillera de Mérida mountain ranges in northern South America (Gemmell and Marcus 2015). This was boundary was described above as the Quito-Barquisimeto line. I produced a graph representing the distance of each collection locality in the proximity of this line (Fig. 4-13 (A)) that included a total of 117 different geographic locations. A statistically significant positive linear relationship was observed ($R^2 = 0.3661$, $p < 0.0001$). Based on our data, the frequency of haplotype group A decreased the further the location was north of the line (to the left, represented by negative distances on the graph) and the frequency of haplotype group A increased the further the location was south of the line (to the right, represented by positive distances). This finding is consistent with previous studies that examined populations in Florida, Cuba and the Bahamas (Lalonde and Marcus 2019a). Initially, all populations were used in this analysis, including populations composed of only a single specimen, creating noise in the data because these populations are mathematically constrained to be either 0% or 100% haplotype group A (Fig. 4-13 (A)). In order to reduce this noise, I removed all locations where the population size was less than 4 specimens to determine if the same relationship was recovered (Fig. 4-13 (B)). This analysis included 60 different geographic locations and had an even stronger significant positive linear relationship ($R^2 = 0.5943$, $p < 0.0001$) than was observed when compared with the first analysis. This linear relationship suggests that the transition in haplotypes from north to south is linear and there is no discontinuity.

**Figure 4-13.** Distance from Quito-Barquisimeto line for all populations (A) and, populations with sample sizes of 4 or larger (B). Each graph includes the best fit linear regression, the $R^2$ value and the p-value.

**A - All populations**



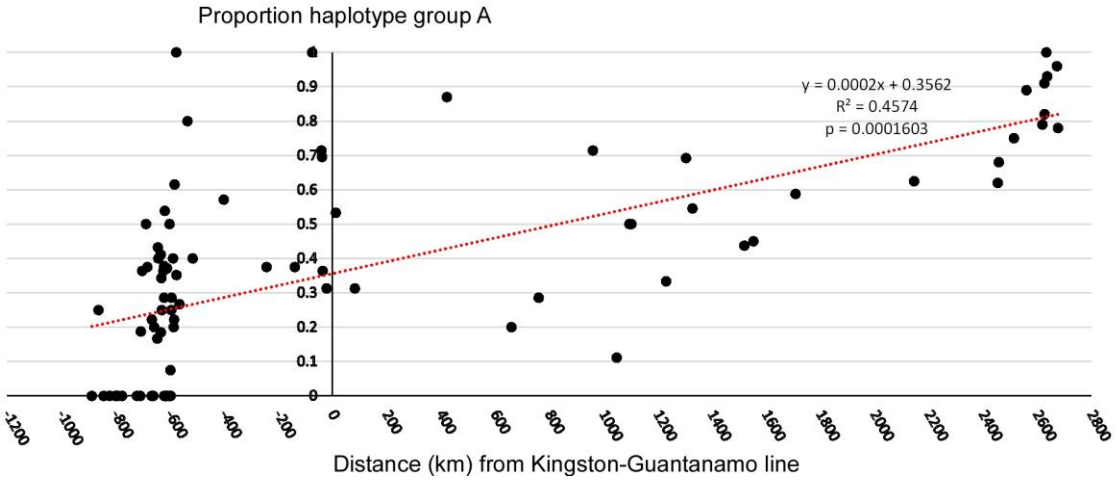

**B - Populations with sample size ≥4 specimens**




A second possible discontinuity in gene flow had been proposed to occur in the Caribbean (Forbes 1928). I plotted a hypothetical discontinuity line that was comparable to the one used for the South American data set, between Kingston, Jamaica and Guantanamo, Cuba. A total of 87 geographic locations were used for this analysis where the sample size in each location was ≥4 only, to reduce noise in the data. A significant positive linear relationship was found ($R^2$ = 0.4574, p =0.0002). Based on our data, the frequency of haplotype group A decreased the further the location was north and west of the line (to the left, represented by

negative distances on the graph) and the frequency of haplotype group A increased the further the location was south and east of the line (to the right, represented by positive distances, Fig. 4-14). The same trend was observed for the South American dataset (Fig. 4-13 (B)). These increases and decreases in proportions of mitochondrial haplotypes in the Caribbean are consistent with our previous work that focused on *Junonia* populations in Florida and the Caribbean (Gemmell and Marcus 2015; Lalonde and Marcus 2019a), suggesting a smooth transition in gene flow in this area and not the abrupt shift that would be expected if a discontinuity exists.

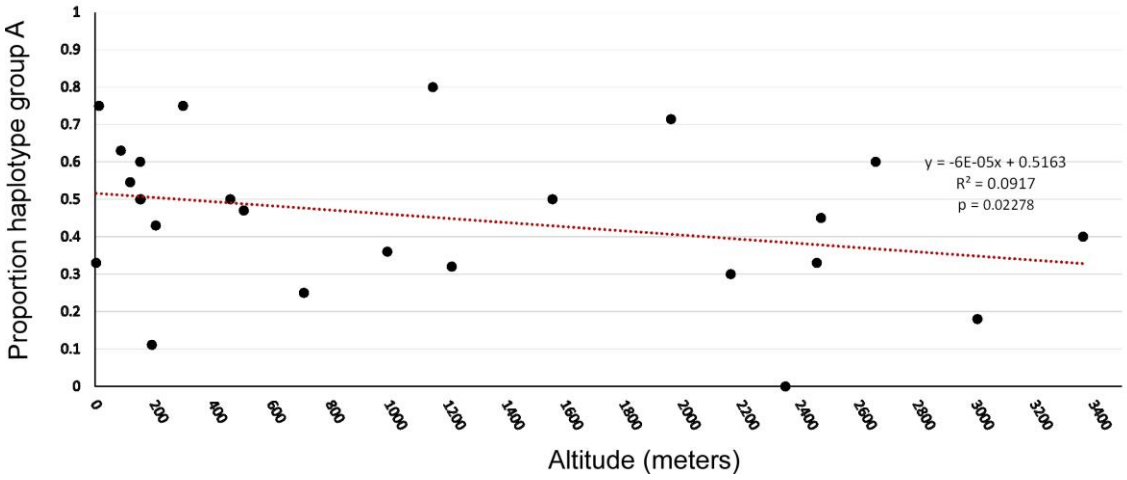
**Figure 4-14.** Distance from Kingston-Guantanamo line for populations with sample sizes of 4 or larger. The graph includes the best-fit linear regression, the $R^2$ value and the p-value.




Previous work showed that there is an apparent association between annual temperature and haplotype group frequencies in *Junonia* populations in Florida (Lalonde and Marcus 2019a). This work showed that there may be a temperature gradient that favours one haplotype over the other, with cooler, more variable temperature regimes most commonly associated with haplotype group B, and warmer, less variable temperature regimes most commonly associated with haplotype group A (Lalonde and Marcus 2019a). The first portion of our study region where a haplotype frequency discontinuity was suspected followed a straight line drawn through the Ecuadoran Northern Andes, Colombian Cordillera Oriental and Venezuelan Cordillera de Mérida mountain ranges in northern South America. As temperature in the region is primarily

determined by altitude, rather than latitude, I tested whether there was an association between elevation and the haplotype group frequency for localities close to the Quito-Barquisimeto line for a total of 25 different locations with a sample size of 4 or larger (Fig. 4-15). Higher elevation localities are considered to be both cooler and harsher environments than lower ones which are considered to be warmer and more stable environments (Hassanin *et al.* 2009; Luo *et al.* 2013). I was able to obtain additional evidence for the higher frequency occurrence of haplotype group B consistent with what was found for Florida (Lalonde and Marcus 2019a). Haplotype group A frequency showed a modest, but statistically significant ($R^2 = 0.0917$, $p = 0.02$) negative correlation with increasing altitude (Fig. 4-15).

**Figure 4-15.** Altitudinal differences in the distribution of haplotype A from populations in the Quito-Barquisimeto data set with populations with sample sizes of 4 or larger. The graph includes the best fit linear relationship, the $R^2$ value and the p-value.




## Discussion

***Junonia* species and their distributions in the Western Hemisphere.** All *Junonia* populations in the Western Hemisphere at one time were considered to be one of two different species: *Junonia evarete* or *J. genoveva* (Forbes 1928; Munroe 1951; Neild 2008; Brévignon and Brévignon 2012; Lalonde *et al.* 2018), both originally described from South America. Two other *Junonia* species, *J. coenia* in North America and *J. vestina* in South America were described in the 19th century, and for the most part, their validity has not been disputed. *Junonia vestina* will

not be discussed further as it was never considered to be part of the *Junonia* ring species (Remington 1985), because it carries unique mitochondrial haplotypes that are not shared with any other species and may be descended from a separate invasion of *Junonia* into the New World (Chapter 5; (McCullagh 2016; Peters and Marcus 2017)).

Since the year 2000 many research groups have contributed to overturning the notion of only four *Junonia* species occurring in the New World (*J. coenia, J. evarete, J. genoveva, J. vestina*). Morphological characteristics, larval host plant use patterns, adult phenology, habitat, and biogeographic data have been used to describe several new cryptic *Junonia* species and to upgrade several subspecies to full species status (Brévignon 2004; Brévignon 2008; Neild 2008; Brévignon 2009; Brévignon and Brévignon 2012; Gernaat *et al.* 2012; Turner and Turland 2017). The documented species distributions for *J. coenia, J. grisea, J. neildi, J. nigrosuffusa, J. litoralis,* and *J. zonalis* had been described in my previous work (Lalonde *et al.* 2018; Lalonde and Marcus 2019b; Lalonde and Marcus 2019a), and have not changed based on additional sampling, but I also include them here for the sake of completeness: *J. coenia* (Fig. 4-12), *J. grisea* (Fig. 4-11 (A)) and *J. nigrosuffusa* (4-11 (B)).

The ranges of three other species have been extended further south due to better geographic sampling in the current study. After consideration of many more *Junonia* specimens, as well as additional molecular characterization of this group (e.g. Cong *et al.* 2020), Pacific coast populations of *J. litoralis* in Mexico and Central America have been reassigned to a newly described species, *J. pacoma* Grishin 2020 (in Cong *et al.* 2020). *Junonia pacoma* has subtle morphological differences that distinguish it from *J. litoralis* that include the subapical patches on the dorsal hindwings being suffused with orange or pink colouration instead of brown, the dorsal forewing anterior eyespot being absent, the anterior dorsal hindwing eyespot being larger than the posterior, a cream coloured antennal shaft, and both mitochondrial and nuclear DNA markers (Electronic Supplement V https://doi.org/10.34990/FK2/I6QUPN/FO5GVH, (Cong *et al.* 2020)). Most of these characteristics where originally interpreted as intraspecific variation of *J. litoralis*. *Junonia pacoma* occurs in Pacific coastal regions of Mexico and Central America (Fig. 4-10 (A)), while thus far *J. litoralis* has only been sampled from Atlantic coastal Panama and French Guiana (Fig. 4-8 (A)). The documented distribution of *J. neildi* has not changed, but subspecies *J. n. varia* Grishin from Texas, USA and Mexico was described (Cong *et al.* 2020) based on its slightly smaller size and its unique combination of nuclear markers (Fig. 4-4 (B)).

We have also documented an extension of the known range of the nominate subspecies of *J. zonalis* into South America to Ecuador, Colombia, Venezuela, and Guyana (Fig. 4-10 (B)). *Junonia z. michaelisi* and *J. z. swifti* remain as restricted island populations (Munroe 1951; Brévignon 2004; Neild 2008).

At least 10 distinct *Junonia* species that are restricted to South America and their distributions will be discussed with respect to continental geography. The arc from Ecuador to the Guyana shield in northern South America was observed to have the highest diversity of *Junonia* species (Fig. 4-2). This is not surprising as this region is known to have some of the highest plant and animal species diversity in the world (Ramirez-Villegas *et al.* 2012; Blandin and Purser 2013), that can be attributed to the combination of altitudinal differences between habitats, which directly affect the distributions of host plants for specialized species, the mountain ranges which can restrict the movement of species and limit migration between habitats, promoting the formation of endemic species, and glacial effects. Of the 10 South American *Junonia* species considered here, all but *J. hilaris* and *J. flirtea* occur in this region. *Junonia hilaris* and *J. flirtea* seem to be restricted to the most southern habitats in our study including Bolivia, Southern Brazil, Argentina, and Paraguay.

**Altitude and *Junonia* haplotypes.** High elevation localities are considered extreme environments where organisms experience physiological challenges associated with low temperature, low oxygen concentrations, and an increase in solar radiation (Blumthaler *et al.* 1997; Hassanin *et al.* 2009; Luo *et al.* 2013). Most research on high elevation organisms is focused on vertebrate species, which have consistently found adaptations associated with respiratory function, therefore changes regarding mitochondrial function ((e.g.,(Torroni *et al.* 2005; Xu *et al.* 2007; Gu *et al.* 2012; Luo *et al.* 2012; Kang *et al.* 2013; Luo *et al.* 2013)). Previous work on *Junonia* populations in Florida associated with mitochondrial haplotype group frequencies and annual temperature showed that there is one mitogenome group that is favoured with less predictable environmental conditions (Lalonde and Marcus 2019a). Haplotype group B was most commonly associated with cooler, and unstable temperature regimes, whereas Haplotype group A tended to be associated with warmer and stable temperature regimes (Lalonde and Marcus 2019a). Cooler and harsher environments with larger temperature fluctuations are characteristic of higher elevation localities, where lower ones are considered to

be more stable and have less variation in temperature therefore more stable environments (Hassanin *et al.* 2009; Luo *et al.* 2013).

In the Northern portion of our South American study area, a group of mountain ranges occurs that is comprised of the Ecuadoran Northern Andes, Colombian Cordillera Oriental and Venezuelan Cordillera de Mérida mountain ranges in northern South America (Blandin and Purser 2013). This area is where a haplotype frequency discontinuity was suspected to exist (Fig. 4-2). To complete distance-based calculations to look for associations with haplotype frequencies (Fig. 4-13), I drew a line through the mountain range which we have named the Quito-Barquisimeto line. Since temperature regimes in mountain ranges is primarily determined by altitude, associations between elevation and haplotype group frequency for closely located localities along this line were analyzed (Fig. 4-15). Haplotype group B in Florida is associated with less stable temperature regimes (Lalonde and Marcus 2019a), which in the case of altitude would be a statistically significant association with high elevation localities, as was observed in South America (Fig. 4-15). Also consistent with previous work done in Florida (Lalonde and Marcus 2019a), the more stable temperature regime localities associated with low elevation, had an association with haplotype group A (Fig. 4-15). This suggests that selection may be occurring for haplotype group B in more harsh environments because haplotype group in *Junonia* is not species specific.

***Junonia* as a ring species.** It has been suggested that ring species provide opportunities to directly observe the process of creating new species and producing biodiversity (Irwin *et al.* 2001; Blackmon and Demuth 2012; Martins *et al.* 2013). The specific criterion that defines a ring species is a ring-shaped distribution, with genetic exchange between populations throughout this distribution with the exception of an area of overlap where no genetic exchange occurs between sympatric populations (Mayr 1963; Joseph *et al.* 2008; Blackmon and Demuth 2012; Gemmell and Marcus 2015). The region of overlap where the interruption in gene flow occurs demonstrates that organisms at the extreme ends of the range have developed reproductive isolation. When gene flow around the ring is interrupted in a second location, this area represents the place where a group of organisms diverges into different species (Mayr 1942; Mayr 1963; Joseph *et al.* 2008; Blackmon and Demuth 2012). Forbes (1928) proposed that the genus *Junonia* was a potential ring species because this genus displays a ring-shaped distribution from Florida,

around the circumference of the Caribbean, across South America, and then into the islands of the Caribbean with a possible discontinuity in gene flow occurring in Cuba (Fig. 4-1 (A)). Forbes (1928) based this apparent discontinuity on morphologically distinct *Junonia* species from the North and South co-occurring in Cuba with no apparent hybridization.

Using the two mitochondrial haplotype groups (A and B) shared across New World *Junonia* species (Pfeiler *et al.* 2012; Gemmell *et al.* 2014; Gemmell and Marcus 2015)., Gemmell and Marcus (2015) tested the prediction of a genetic discontinuity in Cuba as proposed by Forbes (1928). Gemmell and Marcus (2015) determined that no discontinuity was apparent with respect to mitochondrial haplotype frequencies between Cuba and other *Junonia* populations to the North and South. However, they detected a second possible discontinuity at the Isthmus of Panama.

I expanded on this work by sampling *Junonia* much more intensively across North America (nearly 100% haplotype group B, (Lalonde and Marcus 2019b)) and Panama (~75% haplotype group B, (Lalonde and Marcus 2019a)). What remained to be sampled to test the ring species hypothesis was increased sampling of South American *Junonia.* To test if an abrupt transition in gene flow was occurring within this genus, 771 specimens of South American *Junonia* species were obtained, haplotyped, and combined with haplotype data from other parts of the New World for a hemisphere-wide biogeographic analysis.

At first glance, looking across all species (Fig. 4-2), there appears to be a mitochondrial haplotype discontinuity in northern South America, consistent with the predictions of a lack of gene flow near the Isthmus of Panama (Gemmell and Marcus 2015). In the northern portion of South America this discontinuity can be modelled by a line that coincides with the Ecuadoran Northern Andes, Colombian Cordillera Oriental and Venezuelan Cordillera de Mérida mountain ranges (Fig. 4-2), as these mountain ranges can be considered a physical barrier to gene flow for many organisms, including insects. Data from individual species (Figs. 4-2, 4-4 (A), 4-5, 4-6, 4-7 (B), 4-10 (B)) also seems to show the same trend. It should be noted, however, that many of the sampled populations contained very few individuals. At the extreme, a population represented by one individual is mathematically constrained to be either 0% haplotype A or 100% haplotype A. This approach could artefactually distort a linear relationship and make it appear disjunct, so all populations represented by fewer than 4 individuals were excluded from the regression analysis.

When these populations where removed, a linear cline without any major disjunction in haplotype group frequency becomes apparent (Fig. 4-13).

To compare these results to the predictions of the original ring species hypothesis proposed by Forbes (1928), I defined a similar potential discontinuity line in the Caribbean that runs through Cuba, and Jamaica (Fig. 4-14). The slope of this geographic line on the Cartesian plane is identical to that of the Quito-Barquisimeto line that I had defined through northern South America. After removing populations represented by fewer than four samples, a smooth linear cline in haplotype group frequencies was also observed across the Caribbean. This suggests that in both geographic regions, there is no discontinuity in mitochondrial haplotype frequencies or ongoing gene flow, suggesting that the genus *Junonia* fails this to meet this essential criterion for a ring species.

**A general consideration of ring species.** Ring species have been considered a case of evolution in action, through which researchers can observe the creation of new species. Many possible ring species have been proposed, but few have withstood scrutiny. Most do not entirely meet the criteria to be considered a ring species (Irwin *et al.* 2001; Liebers *et al.* 2004; Joseph *et al.* 2008; Kuchta *et al.* 2009; Blackmon and Demuth 2012; Cacho and Baum 2012; Martins *et al.* 2013; Kuchta and Wake 2016). The three best examples that remain, the herring gull complex (Mayr 1942; Mayr 1963; Liebers *et al.* 2004), the greenish warbler complex (Wake and Yanev 1986; Price *et al.* 1997; Irwin *et al.* 2001; Martins *et al.* 2013), and the salamander complex from California (genus *Ensatina*) (Wake and Yanev 1986; Wake *et al.* 1986; Moritz *et al.* 1992; Kuchta *et al.* 2009), have biogeographical species distributions in the shape of a ring, have morphologically similar species that occur around the distribution, and have a portion where the ends of the ring meet where gene flow does not occur.

Why most proposed ring species do not meet the criteria of a true ring species concerns the genetic exchange between the populations that reside around the distribution, and not in the region of apparent discontinuity in gene flow. In the case of the herring gulls (genus *Larus*) (Mayr 1942; Mayr 1963), although an area of discontinuity in Northern Europe where sympatric populations that are reproductively isolated is present, based on molecular data these two species did not come from the same refugia (Liebers *et al.* 2004). Thus, gene flow around the ring does not form a continuous gradient from a single source population but instead comes from two

separate lineages (Liebers *et al.* 2004). The greenish warblers (genus *Phylloscopus*) (Mayr 1963) are the most widely accepted example of a ring species (Price *et al.* 1997; Irwin 2000; Irwin *et al.* 2001; Irwin 2002; Blackmon and Demuth 2012). The warblers have sympatric populations in the region of overlap that are reproductively isolated (in Siberia in this case (Price *et al.* 1997; Irwin 2000; Irwin *et al.* 2001; Irwin 2002)), but an additional large gap in gene flow occurs in China due to a habitat interruption due to deforestation (Irwin 2002). Due to this large gap in China (Irwin 2002), a continuous gradient in gene flow is lacking on both sides of the ring, even though both lineages originate from a single source population (Price *et al.* 1997). The salamanders (genus *Ensatina*) are a third plausible ring species, displaying a morphological gradient with a freely interbreeding population in Northern California, and in the south have sympatric populations that do not interbreed (Wake and Yanev 1986; Wake *et al.* 1986; Highton 1999; Kuchta *et al.* 2009; Blackmon and Demuth 2012; Kuchta and Wake 2016). An additional discontinuity in gene flow and morphology occurs in a region called Lassen Peak in the northern part of the range so gene flow is not continuous on the eastern part of the ring (Wake *et al.* 1986; Highton 1999; Blackmon and Demuth 2012). All of these examples fail to meet at least one criterion, continuous gene flow, to classify them based on Mayr's (1963) definition of ring species.

The New World *Junonia* species fail to meet the criteria of a ring species in a different way than the examples explained above. Although their distribution in the New World has a ring distribution around the Caribbean, and a transition in mitochondrial haplotypes seems apparent based on both previous studies (Gemmell *et al.* 2014; Gemmell and Marcus 2015; Lalonde and Marcus 2020a) and this study (Fig. 4-2), additional analysis using distance calculations from two different possible discontinuous boundary lines (Fig. 4-13, Fig. 4-14) show that gene flow is continuous and homogenized based on mitochondrial genome data suggesting that substantial gene flow is taking place between the species in this genus, even in the supposed region of overlap. Therefore, no interruption in gene flow is occurring in the New World falsifying all previous hypotheses suggesting *Junonia* is a ring species in this biogeographic region.

Since no good examples that clearly meet all criteria to be considered a ring species in the real word exist, one may ask how useful is the concept of ring species? The concept of ring species is still useful as it allows for the exploration of the processes of speciation by focusing on how models based on allopatric speciation may work, based on isolation by distance of discrete

populations, which is considered to be one of the most effective ways to explain the divergence of species (Mayr 1942; Mayr 1963). It allows researchers to study and explore how biogeography plays a role in the diversification of species and observe how gene flow may change across the range of an organism, by using space as a surrogate for time, as we usually cannot directly observe time (Irwin *et al.* 2001; Joseph *et al.* 2008; Blackmon and Demuth 2012; Kuchta and Wake 2016). As most proposed ring species occur across a large geographic range that surrounds unsuitable habitat, species can be studied as closed genetic systems, and be used to investigate how species diversity may have arisen over time (Joseph *et al.* 2008; Blackmon and Demuth 2012; Kuchta and Wake 2016). Other questions one may pose is whether true ring species do not exist because they are not important in the context of the evolution of species groups? Or maybe it is it that ring species are just unstable intermediate states of the speciation process that do not last long enough to be detected while they are still intact? This unstable intermediate state of speciation may last only for a very short period of time such that researchers may not have the means to observe them, due to a rapid and recent diversification, recently termed as ring ephemeral species (Bouzid *et al.* 2021). This makes them important evolutionary model systems that can be used to attempt to answer important evolutionary questions.

Recent work exploring the diversification patterns using the western fence lizards, provides insight into what an ephemeral ring species is (Bouzid *et al.* 2021). This example shares many similarities with the diversification patterns we see in the buckeye butterflies.. Based on genetic data, the western fence lizards are thought to have expanded northward from southern refugia after glaciation period to suitable habitats surrounding the unsuitable Sierra Nevada and Cascade Mountain ranges. This created a ring-shaped distribution (Bouzid *et al.* 2021). Gene flow around the ring occurs between closely situated populations, but with reproductive isolation incomplete in the northern potions of their range (in the area of overlap where formal ring species definitions would place a gene flow discontinuity) thought to be caused by secondary contact (Bouzid *et al.* 2021). The rapid expansion of ranges of species may allow for previously allopatric populations to become sympatric, and if secondary contact allows hybridization between populations it can be a means of a quick radiation of species, increasing species diversity in groups very rapidly (Seehausen 2004; Heliconius Genome Consortium 2012; Nichols *et al.* 2015). This recent and rapid range expansion followed by hybridization in the Western fence lizards has produced an adaptive radiation, increasing both the biodiversity and

geographic range, as they recover from range contraction due to glaciation (Cristescu 2015; Nichols *et al.* 2015).

The patterns of species diversification in North America and Cuba associated with *Junonia* range expansion after periods of glaciation and the creation of secondary contact zones after the Pleistocene, has already been characterized in this group (Lalonde and Marcus 2019a; Lalonde and Marcus 2019b). As *Junonia* is thought to have invaded North America from South America (Gemmell and Marcus 2015), it also mirrors this Northern expansion from this point around unsuitable habitat, the Caribbean Sea and Gulf of Mexico. What has not been taken into consideration in this genus is possible *Junonia* range contraction in South America during the Pleistocene glaciation, which also plays a key role in the diversification of this genus in the New World. The Pleistocene glaciation in South America included the entirety of the Andes Mountain range (including the Ecuadoran Northern Andes, Colombian Cordillera Oriental and Venezuelan Cordillera de Mérida mountain ranges), with Patagonian ice sheets extending down onto the valleys within South America (Glasser *et al.* 2008), with the species that occur in this region receding into various glacial refugia in the South American Basin and the closely associated islands of the Caribbean and Panama. Once the glacial ice sheets receded, it would have allowed for secondary contact of many different species emerging from glacial refugia. Based on our characterization of *Junonia* in South America, the largest number of New World species occur in this region. If South America was the original point of New World colonization for *Junonia*, there would have been more time for the diversification of species to occur there compared to the rest of Western Hemisphere. With secondary contact after glacial recession, hybridization among lineages emerging from refugia may have caused an explosion of species to occur in this region, generating even more genetic diversity in this group. This pattern of diversity is consistent with what Bouzid et al. (2020) observed using the western fence lizards, so it is possible that although *Junonia* do not entirely fit the definition of a ring species (Mayr 1942; Cain 1954; Mayr 1963) due to ongoing gene flow, it may better fit the definition of an ephemeral ring species (Bouzid *et al.* 2021). It is possible that what we are observing is a stage in the process of speciation, due to its rapid and recent diversification and that not enough time has passed for lineage coalescence to occur.

## Conclusion

Ring species are rare and none of the classic examples meet all of the criteria such that they would be classified as being a true ring species, including the New World *Junonia*. In many examples, this is due to multiple gaps in gene flow around the ring, while in *Junonia* butterflies there is continuous gene flow with no gaps. New World *Junonia* are a recently diverged group, so hybridization, and gene flow are ongoing among many New World populations. This continuous gene flow is consistent with a recent example, termed an ephemeral ring species (Bouzid *et al.* 2021), which may represent a process in speciation that may occur because not enough time have passed for a discontinuity to form because of a rapid and recent diversification. While the *Junonia* ring species hypothesis has been falsified using the classical definition, this work has answered important questions regarding the number of species, and subspecies that occur in the New World and has mapped their geographic distributions for the first time.

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## Connecting section: Chapter 4 to Chapter 5

In Chapter 4, I falsified the New World *Junonia* ring species hypothesis due to ongoing gene flow in Panama and northern South America as well as across the Caribbean. With the help of new additional sampling, I was able to document 18 *Junonia* species in the New World, compile a table of morphological and life history data for the delimitation of these species, and characterize geographic ranges for each of these species, with the exception of *J. vestina* which carries its' own unique mitochondrial haplotype group.

Chapter 5 will focus on creating the most comprehensive molecular phylogenies to date for the genus *Junonia* and the tribe Junoniini. As the New World *Junonia* represent a recent radiation with many shared mitochondrial haplotypes in the DNA barcode region across species, I will use the full mitogenome sequences and complete nuclear rRNA repeats to generate distinct large sequence data sets with strong mitochondrial and nuclear phylogenetic signals. This should alleviate some the previous challenges in producing well-resolved phylogenetic trees associated with this group. I will also use these phylogenetic reconstructions to address hypotheses related to long standing research questions for this group, including the sister taxon to genus *Junonia*, as well as their most probable route of colonization of the New World by *Junonia.*

# Chapter 5: A global molecular phylogeny and insights on the dispersal and invasion history of the genus *Junonia*.

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Author Contributions:

ML designed the study, did data collection, performed experiments, analyzed data and drafted the manuscript.

JM assisted with study design, provided laboratory space, research materials, and revisions to the manuscript.

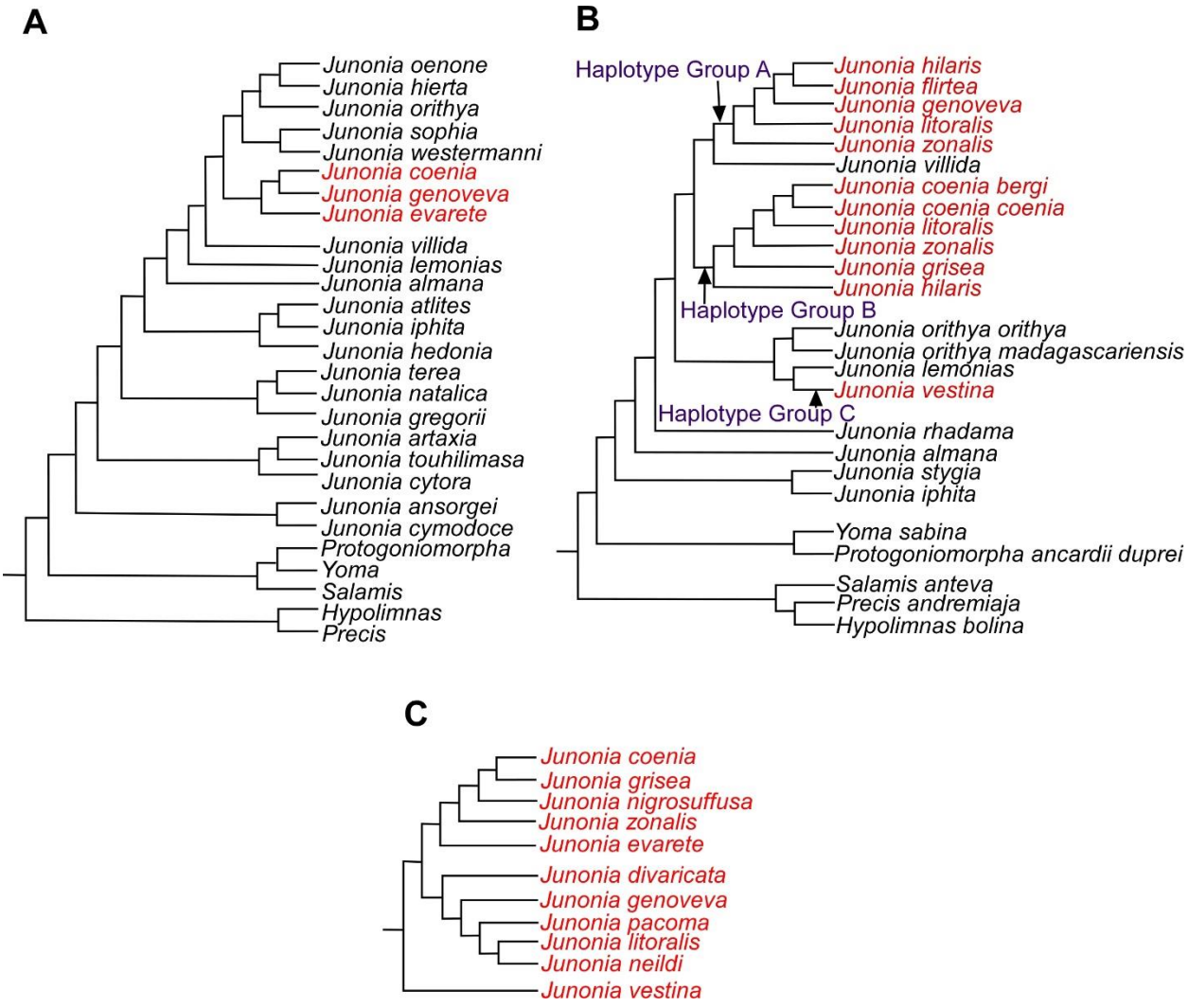
**Abstract**

Many disparate hypotheses have been proposed for the phylogenetic relationships within the butterfly genus *Junonia* (Nymphalidae), yet there remains no consensus. Most phylogenetic hypotheses have been based on morphology or on DNA sequences from only a small number of mitochondrial and nuclear genes, and all have suffered from incomplete taxon sampling, focusing primarily on either New World or Old World species. To further exacerbate the phylogenetic ambiguities that exist in this genus, there have been taxonomic disputes involving several species known by the same name simultaneously, as well as mitochondrial haplotype groups that are shared across all New World species in this genus. There is consensus that the genus *Junonia* originated in Africa but whether their route into the New World was trans-Atlantic or trans-Pacific is still unknown. Here I have generated the largest comprehensive phylogeny of the genus to date, using full mitochondrial genomes and the rRNA repeat region of the nuclear genome (including the 18S, 28S, and 5.8S rRNAs and the Internal Transcribed Spacers ITS1 and ITS2) from 40 of the 47 described *Junonia* species. Based on these phylogenetic results, one can conclude that *Junonia* is monophyletic and the most probable sister clade to *Junonia* appears to be genus *Salamis*. There is some evidence of ongoing genetic exchange between *J. villida* from the Indo-Pacific and the New World *Junonia* (particularly *J. vestina*), perhaps suggesting that genus *Junonia* entered the New World via a trans-Pacific route, which is consistent with some prior biogeographic hypotheses. However, in both the mitogenome and the nuclear rRNA repeat phylogenetic analyses, the sister clades to the bulk of the New World *Junonia* contains species that occur in both Africa and Asia. This raises the possibility that more than one *Junonia* invasion may have contributed to the New World diversification, and dispersal may have taken place across either the Atlantic or the Pacific Oceans. Finally, it appears that hybridization and lateral transfer of mitochondrial genomes between lineages, a phenomenon already well documented in New World *Junonia* taxa, may also occur in at least 2 Old World lineages (*J. orithya*/*J. hierta* and *J. iphita*/*J. hedonia*). The additional genetic variation associated with this genetic exchange between lineages may have contributed to the patterns of speciation and diversification observed in this genus.

## Introduction

The genus *Junonia* has had a convoluted taxonomic history, especially for species that occur in Western Hemisphere. This can be attributed to interchangeable use by some authors of the generic names *Precis* (properly restricted to a related clade in Africa) and *Junonia* (which includes species from every continent except Europe and Antarctica), misidentifications due to intraspecific seasonal and geographical variation in morphological characteristics, loss or absence of type specimens, and the failure of many authors to make the explicit reference to taxonomic authorities they have used to assign names to specimens in their published work (Neild 2008; Brévignon and Brévignon 2012; Lalonde *et al.* 2018). Due to these ambiguities, creating a robust phylogeny for the genus *Junonia* has been difficult. Several morphology-based phylogenetic hypotheses have been proposed (Fig. 1-1 (A, B, C)), but have yet to be broadly accepted because they are contradictory. They also differ from the available molecular phylogenies based on a small number of mitochondrial (especially DNA barcode sequences) and nuclear genes (Fig. 1-1. (D, E), Fig. 5-1). Finally, another contributing factor is that in the New World, mitochondrial DNA haplotypes tend to be shared among all sympatric species in any given geographic location, resulting in identical mitochondrial gene sequences (Pfeiler *et al.* 2012; Gemmell *et al.* 2014; Gemmell and Marcus 2015; Lalonde *et al.* 2018; Lalonde and Marcus 2019c). Originally the mitochondrial haplotype groups were defined using the DNA barcoding sequence (~658bp portion of *cytochrome c oxidase subunit I*) as a marker, but recent work has shown that all New World *Junonia* mitogenomes show similar patterns of near-identity in all mitochondrial genes based on haplotype group (Peters and Marcus 2017).

**Figure 5-1.** Recent molecular phylogenies of the genus *Junonia*. A) based on Kodandaramaiah (2009; Figure 4) composed of 2 nuclear genes (*Ef1 alpha*, *wingless*) and partial mitochondrial *COI* genes. Note that this representation is the same tree used by Clarke (2017) to trace the evolution of morphological character states. B) based on Lalonde & Marcus (2020c; Figure 1) analysis of complete mitochondrial genomes. C) based on Cong et al. (2020; Figure 16b) analysis of Z chromosome sequences (nuclear genome). New World *Junonia* taxa are represented in red.



Taxonomic assignments based on morphology have become less difficult in recent years due to work clarifying taxonomic designations for species in the New World (Brévignon 2004; Brévignon 2009; Brévignon and Brévignon 2012; Gemmell *et al.* 2014; Lalonde *et al.* 2018; Lalonde and Marcus 2019c; Cong *et al.* 2020) but still remain challenging for the reasons described above, as well as by the existence of undescribed, poorly described, or cryptic *Junonia* species (Lalonde and Marcus 2019c; Cong *et al.* 2020). In general, *Junonia* phylogenetic studies have not considered the genus as a whole. Instead, they present phylogenetic tree reconstructions containing either very few New World species (Fig. 1-1 (D, E), Fig. 5-1 (A), (Kodandaramaiah 2009; Clarke 2017; Pyrcz *et al.* 2021)), or lacking Old World species (Fig. 5-1 (C), (Cong *et al.* 2020). For example, Clarke (2017) included a single New World species (*J. coenia*) in their study, Kodandaramaiah (2009) included three New World species (Fig. 5-1 (A)), and Cong et al. (2020) included no Old World species (Fig. 5-1 (B)). Further, some phylogenetic studies of New World taxa have included sequences where the identifications of the source specimens were either wrong or ambiguous. For example, Pfeiler et al. (2012; Fig. 1-1 (E)) used many sequences mined from GenBank, which were labeled as either *J. evarete* or *J. genoveva* in the database, and it is still unclear if the identity of all of the source-specimens are indeed as labeled, or if they belong to one of six other potential *Junonia* species (see Chapter 4) as few of these specimens were identified with confidence and museum vouchers are not available.

More recently, the use of DNA barcoding (Kodandaramaiah and Wahlberg 2007; Pfeiler *et al.* 2012; Gemmell *et al.* 2014; Gemmell and Marcus 2015) and later, full mitochondrial genome sequencing (Peters and Marcus 2017; Lalonde and Marcus 2019b; Lalonde and Marcus 2020c; Living Prairie Mitogenomics Consortium 2020), have determined that *Junonia* is a monophyletic clade, but that the New World *Junonia* are not completely monophyletic due to a complicated evolutionary history likely involving multiple invasion events into the New World followed by hybridization between invading lineages (McCullagh 2016; Peters and Marcus 2017). These studies have provided increased resolution that was lacking in earlier molecular phylogenetic reconstructions, although many *Junonia* species are still missing from these analyses (eg. Fig. 5-1 (B)). The invasion events into the New World have been the subject of speculation by researchers for many decades. There is now consensus that the genus *Junonia* originated in Africa (Kodandaramaiah and Wahlberg 2007; Pfeiler *et al.* 2012), but which lineages invaded the New World to give rise to the species that now occur there remains an open

question. At least four separate taxa have been suggested as the sister taxa to the New World *Junonia*: *J. villida* from the Indo-Pacific (Forbes 1947), *J. orithya madagascariensis* from Africa (Eliot 1946; Eliot 1947), *J. orithya orithya* from Asia (Corbet 1948), and *J. lemonias* from Asia (McCullagh 2016; Peters and Marcus 2017). In addition, it is also a matter of debate if the occupation of the New World by *Junonia* was a unique event (Forbes 1928; Eliot 1946; Eliot 1947; Forbes 1947; Kodandaramaiah and Wahlberg 2007; Neild 2008; Pfeiler *et al.* 2012) or was caused by multiple invasions involving one or more Old World species (Gemmell and Marcus 2015; McCullagh 2016). Recent full mitochondrial genome sequence data suggests a trans-Pacific route is more probable and it is possible that the same lineage (*J. villida*) crossed the Pacific more than once to reach the New World (McCullagh 2016; Peters and Marcus 2017).

Based on previous studies, individual Old World *Junonia* have generally been found to be monophyletic based on both molecular and morphological data (Wahlberg *et al.* 2005; Kodandaramaiah and Wahlberg 2007; Kodandaramaiah 2009; Clarke 2017). In contrast, New World *Junonia* species do not form monophyletic clades based on most molecular studies (Pfeiler *et al.* 2012; Gemmell *et al.* 2014; Gemmell and Marcus 2015; McCullagh 2016; Peters and Marcus 2017; Lalonde *et al.* 2018; Lalonde and Marcus 2019c; Cong *et al.* 2020; Lalonde and Marcus 2020c). Instead, all New World *Junonia* species tend to share mitochondrial haplotypes and populations of each *Junonia* species in any particular geographic location typically show the same mitochondrial haplotype group frequencies (Gemmell and Marcus 2015; Lalonde and Marcus 2020a; Lalonde and Marcus 2020b). It has been observed that many New World *Junonia* species have the capacity to interbreed, so hybridization and mitochondrial introgression events do occur (Hafernik 1982; Paulsen 1996; Lalonde and Marcus 2019c; Cong *et al.* 2020). Consequently, mitochondrial gene-based phylogenies are not useful for New World species delimitation (Kodandaramaiah and Wahlberg 2007; Kodandaramaiah 2009; Pfeiler *et al.* 2012; Gemmell *et al.* 2014; Gemmell and Marcus 2015; Peters and Marcus 2017; Lalonde *et al.* 2018; Lalonde and Marcus 2019c; Cong *et al.* 2020), but if determination of species is done using other characteristics, the study of mitochondrial haplotype distributions allows for the characterization of biogeographic patterns of genetic variation ((Gemmell and Marcus 2015; Lalonde and Marcus 2019c; Lalonde and Marcus 2020a) Chapter 4).

**DNA Barcodes, short fragment nuclear DNA, and haplotype groups.** In the New World there are three primary mitochondrial haplotype groups (two of which can be subdivided into subgroups) that are shared by many species (Fig. 5-1) (Kodandaramaiah and Wahlberg 2007; Kodandaramaiah 2009; Pfeiler *et al.* 2012; Borchers and Marcus 2014; Gemmell *et al.* 2014; Gemmell and Marcus 2015; Lalonde *et al.* 2018; Lalonde and Marcus 2019c; Cong *et al.* 2020). This pattern was first discussed by Pfeiler et al. (2012), who used the 658 base pair DNA barcoding fragment of the *cytochrome c oxidase I* (COI) gene, which has been useful for delimiting species in many other animal taxa (Hebert *et al.* 2003a; Hebert *et al.* 2003b). Pfeiler et al. (2012) suggested that this pattern of shared mitochondrial haplotypes may be due to the recent invasion of *Junonia* into the New World (~2–4 million years ago) (Kodandaramaiah and Wahlberg 2007), such that lineage sorting of species is incomplete and hybridization between species is ongoing. Pfeiler et al. (2012) also suggested that it is possible that a single polytypic species invaded the New World. Although they found that species delimitation was not possible in this group using DNA barcodes and that the New World *Junonia* species shared two distinct mitochondrial haplotype groups (diverged ~2.2 million years ago), which formed monophyletic clades (Pfeiler *et al.* 2012). Haplotype group A that is most commonly found in species from South America and the Caribbean, and Haplotype group B is most commonly found in species from North and Central America (Pfeiler *et al.* 2012). They also suggested that *J. vestina* (a high elevation species) had itself a distinct sequence that they called haplotype $A_1$, and considered all other species to carry $A_2$ (Pfeiler *et al.* 2012). They did not subdivide haplotype group B but did note that *J. coenia grisea* in California carried a unique version of this haplotype group. Based on their choice of outgroups, Pfeiler et al. (2012) speculated that the sister taxon to the New World *Junonia* may have been from Africa and from a lineage related to either *J. orithya* or *J. westermanni*, although this was based on very limited sampling of Old World species (Fig. 1-1 (E)).

Borchers and Marcus (2014) published results that were largely congruent with Pfeiler et al. (2012), but with some differences due to more extensive taxon sampling. They found that the New World *Junonia* were apparently paraphyletic based on molecular data, but that each of the main haplotype groups were monophyletic. They discovered that haplotype group B also occurs in some *Junonia* populations in Argentina, and that this haplotype group is more similar to some Old World mitochondrial genomes (mitogenomes) than they are to haplotype group A (Borchers

and Marcus 2014). They hypothesized that the two haplotype groups in the New World may be the result of an ancient mitogenome polymorphism present in the original founding *Junonia* populations (Borchers and Marcus 2014). Similarly, Gemmell et al. (2014) obtained results comparable to Pfeiler et al. (2012) based on the distribution patterns of haplotype groups, with group A being most frequent in South America and the Caribbean, and B most prominent in North America, Central America, the Caribbean. Gemmel et al. (2014) also found haplotype group B to occur at very low frequency in South America, as Borchers and Marcus (2014) had. Their work also showed that *J. vestina* and *J. flirtea* consisted solely of haplotype group A (Gemmell *et al.* 2014).

Like earlier studies, Gemmell et al. (2014) found the New World *Junonia* to be paraphyletic, but the haplotype groups that occur within these species were monophyletic. Haplotype group B was the only haplotype that was found in most North American populations of *J. coenia* and *J. nigrosuffusa.* They also suggested that the South American taxon *J. flirtea*, may contain two morphologically cryptic species (Gemmell *et al.* 2014), a possibility which to date has not been explored further. Gemmell and Marcus (2015) mapped the distribution patterns of haplotype groups in the New World and confirmed that *J. coenia grisea* from California had a distinct variant of the B haplotype group. Gemmell and Marcus (2015) also suggested that mitochondrial DNA sequences from *J. villida* in the Indo-Pacific are more similar to the New World *Junonia* than sequences from *J. orithya*, which is consistent with some previous hypotheses based on morphology and geographic distributions (Seitz 1914; Forbes 1928; Vane-Wright and Tennent 2011). Gemmell and Marcus (2015) proposed two hypotheses for the origin of haplotype B: either it evolved from haplotype group A mitochondria within the New World, or that two separate invasions into the New World carried haplotype groups A and B, followed by hybridization between species descended from the two invasion events.

My work on *Junonia* began in 2014 and my early studies were restricted to the New World *Junonia*, which are all open habitat or grassland species. I was able to determine that three distinct species occur in Florida (Lalonde *et al.* 2018). In addition, I found that the species that were being referred to as *J. evarete* and *J. genoveva* in Florida were not correctly identified. The mangrove buckeye (formerly *J. evarete*) is properly called *J. neildi*, and the tropical buckeye (formerly *J. genoveva*) was in fact *J. zonalis* (Lalonde *et al.* 2018; Lalonde and Marcus 2019a). Both species are common in the Caribbean. I also determined that the true *J. genoveva* and *J.*

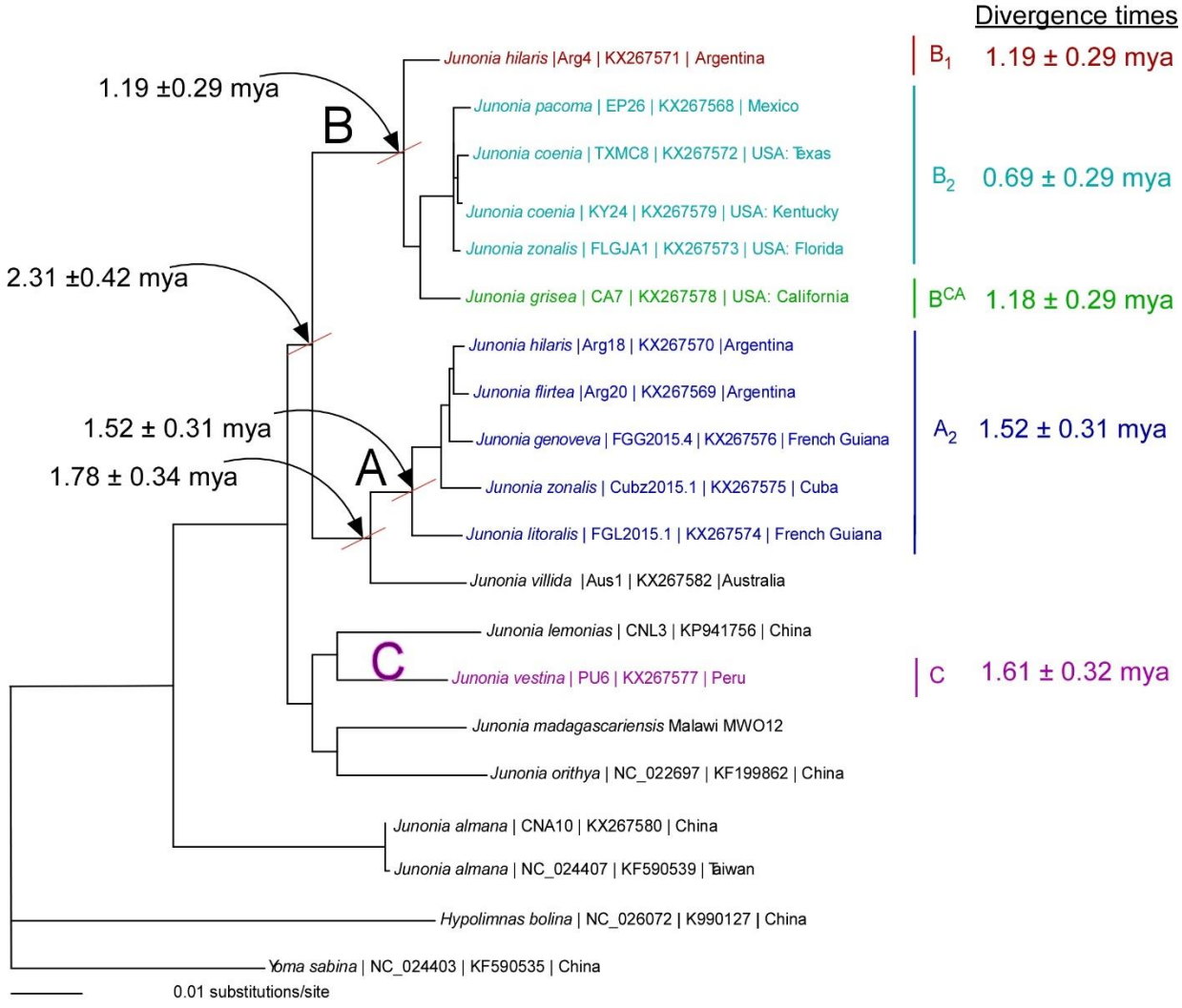
*evarete* only occur in South America, so the *Junonia* populations that were being labelled as these taxa in Central America, Mexico, North America, and the Caribbean had been incorrectly identified in previous work (Lalonde *et al.* 2018). I was able to determine that unlike most of North America, haplotype group A is found in Florida in two *Junonia* species (*J. neildi* and *J. zonalis*) (Lalonde *et al.* 2018). Later, I discovered that some individuals of the third species in Florida, *J. coenia*, also had haplotype group A, although at very low frequency (Lalonde and Marcus 2020a). The sharing of haplotype groups between the *Junonia* species has been attributed to naturally occurring hybridization and introgression events (Hafernik 1982).

Next, I focused on the taxonomy of *Junonia* species in the American Southwest and Mexico. I determined that five *Junonia* species occur in this region and that hybridization occurs readily between these species (Lalonde and Marcus 2019c). The distinct group B haplotype associated with *Junonia* specimens from California, which is now known as haplotype $B^{CA}$, was found to be the principle mitochondrial haplotype of a cryptic taxon, considered by some to be a subspecies (Austin and Emmel 1998), which was elevated to a full species status: *J. grisea*, (Lalonde and Marcus 2019c). This work also showed that the haplotype $B^{CA}$ was not restricted to *J. grisea* or even to California, and was found at low frequency in the other 4 species in the American Southwest (Lalonde and Marcus 2019c). It is important to note that the molecular analysis underlying these findings used a small fragment of the mitochondrial *COI* gene (DNA barcoding), and/or few nuclear markers (e.g., Randomly amplified fingerprint data (RAF), *wingless*, *Elongation factor-1-alpha*).This work has given valuable insights into the relationships of the *Junonia* species in the New World and has been the foundation for the work I will be discussing below that includes the most comprehensive molecular phylogenies using both the whole mitogenome and the rRNA repeat, in an attempt to answer where the genus *Junonia* originated, and which species was responsible for the New World radiation of the genus.

**The utility of full mitochondrial DNA genomes.** Full mitochondrial genome sequences have been a rich source of genetic variation for phylogenetic studies (Hao *et al.* 2013; Gillett *et al.* 2014; Timmermans *et al.* 2014; Wu *et al.* 2014). Lepidopteran mitogenome datasets contain more than 15,000 base pairs including many phylogenetically informative characters that have helped increase taxonomic resolution in some groups (Hao *et al.* 2013; Gillett *et al.* 2014; Timmermans *et al.* 2014; Wu *et al.* 2014; Shi *et al.* 2015). The first two full mitochondrial

genome sequences reported for the genus *Junonia* were both from Old World species (Wu *et al.* 2014; Shi *et al.* 2015). Subsequently, a total of 14 complete New World *Junonia* genomes and four additional Old World mitochondrial genomes were assembled and used to reconstruct patterns of diversification of *Junonia* species (Fig. 5-2, (McCullagh and Marcus 2015; McCullagh 2016)).

**Figure 5-2.** Molecular phylogeny of the genus *Junonia* using full mitochondrial genomes from McCullagh (2016). Haplotypes and their divergence times are included.



This work confirmed that subfamily Nymphalinae and genus *Junonia* form monophyletic groups, but like previous findings using *COI* sequences, the New World *Junonia* were not monophyletic. Multiple *Junonia* invasion events into the New World is probable (Fig. 5-2, (McCullagh 2016)), as had been previously proposed based on smaller data sets (Gemmell and Marcus 2015). It was suggested that separate invasions by different Old World species may have created each of the New World haplotype groups (Fig. 5-2, (McCullagh 2016; Peters and Marcus 2017)). The haplotype groups A and B in *Junonia* was estimated to have diverged 2.31± 0.42 million years ago (mya), which is consistent with previous molecular clock estimates (Kodandaramaiah and Wahlberg 2007; Pfeiler *et al.* 2012). McCullagh (2016) also estimated divergence times for all other haplotype groups in the New World (Fig. 5-2): the divergence of $A_1$ and $A_2$ was estimated to be 1.52±0.31 mya; the divergence of $B_1$ from the rest of haplotype B was estimated to be 1.19±0.29 mya; and the divergence the *J. vestina*-restricted haplotype that is now known as haplotype group C (Peters and Marcus 2017) was estimated to be 1.16±0.32 mya. The work of McCullagh (2016) paved the way for additional and more comprehensive analyses of full *Junonia* mitogenomes, to which I have added analysis of an ~8,000 bp fragment of another highly abundant sequence in the genome, the nuclear rRNA repeat, which contains three rRNA repeat subunits (2.8S, 18S, 28S) and two internal transcribed spacers (ITS1, ITS2).

Since the work of McCullagh (2016), many additional mitochondrial genome sequences have become available for *Junonia* and other species within the Tribe Junoniini. Peters and Marcus (2017) used full mitogenome sequences and other markers to test for evidence of gene flow between an endemic *Junonia coenia* subspecies in Bermuda and other *Junonia* populations in North America. They determined that gene flow between Bermuda and the mainland is ongoing and that the *Junonia* of Bermuda should not be elevated to a full species. They also recognized the distinctiveness of New World haplotype group C that is restricted to a single high elevation species, *J. vestina*, which is more closely related to *J. lemonias* from Asia than it is to other New World Haplotypes (Peters and Marcus 2017). Consistent with previous studies, haplotypes A and B each formed monophyletic groups (Pfeiler *et al.* 2012; Borchers and Marcus 2014; Gemmell *et al.* 2014; Gemmell and Marcus 2015; McCullagh 2016), but the placement of haplotype group C makes the New World *Junonia* paraphyletic (Peters and Marcus 2017).

Recently, Cong et al. (2020) analyzed Next-generation sequencing libraries from 11 New World *Junonia* species (but did not examine Old World *Junonia* species) to describe two new

species (*J. pacoma*; the Pacific mangrove buckeye and *J. stemosa*; the South Texas dark buckeye). They could not identify any fixed diagnostic characters (morphological or molecular) that allow for the consistent separation of *J. stemosa* from the morphologically nearly identical *J. nigrosuffusa,* so I will treat this form as a subspecies *J. nigrosuffusa stemosa* **nov. stat.** (Fig. 5-1 (C), (Cong *et al.* 2020)). The reason for this choice is that based on the work by Cong et al (2020) *J. n. stemosa* represents a hybrid lineage (between *J. nigrosuffusa* and *J. grisea*) so it is not an independent evolutionary lineage, it has no diagnostic morphologically characteristics to distinguish it from *J. nigrosuffusa*, does not exhibit any form of reproductive isolation, and thus does not satisfy the requirements of what should be considered a full species for this genus (Peters and Marcus 2017).

The most comprehensive mitogenome-based phylogeny of the genus *Junonia* with extensive outgroup species from the tribe Junoniini was published in 2020 consisting of 28 mitogenomes from Junoniini (including15 *Junonia* species (8 New World, 7 Old World), and one sequence from each of the five other genera) (Fig. 5-1 (B), (Lalonde and Marcus 2020c)). This analysis concluded that *Junonia* is a monophyletic clade consistent with the previous studies mentioned above, haplotype groups A and B form monophyletic clades, the New World *Junonia* is not monophyletic, and the most likely sister clade to genus *Junonia* (though with weak bootstrap support) contains both African and Asian genera (*Yoma* and *Protogoniomorpha*), all of which is consistent with a recent analysis of mitochondrial DNA barcodes from Old World *Junonia* species (Pyrcz *et al.* 2021).

There are now many mitogenomes available for *Junonia*, and other genera from the tribe Junoniini through GenBank: 18 of the 47 *Junonia* species and single sequences from each of the five other genera (Table 5-1). Yet, mitogenomes from other species are still missing and a comprehensive phylogenetic analysis of the genus is not yet possible to address the question of which lineages or species are responsible for the *Junonia* invasion events into the New World. Here I build upon this pre-existing knowledge by assembling and incorporating additional full mitochondrial genomes from the genus *Junonia* (22 new species, for a total of 40 of the 47 described species), the tribe Junoniini (16 additional species distributed across the 5 genera), and additional outgroup species into a phylogenetic analysis. To complement this mitogenome-based phylogeny, I conducted a phylogenetic analysis based on the nuclear rRNA repeats from the

same group of samples and species in order to better delimit species and understand the relationships between them.

**Table 5-1**. The 47 *Junonia* species (with subspecies) sorted by New World and Old World. A * indicates species used in current molecular analysis.

| Species |
|---|
| **New World *Junonia*** |
| *J. coenia* Hübner, 1822 |
| *J. coenia bergi* Avinoff, 1926 * |
| *J. coenia coenia* Hübner, 1822 * |
| *J. divaricata* C. & R. Felder, 1867 |
| *J. divaricata divaricata* C. & R. Felder, 1867 * |
| *J. divaricata houlberti* Brévignon, 2008 |
| *J. divaricata mitraka* Brévignon, 2012 |
| *J. evarete* Cramer, 1779 |
| *J. evarete dougueti* Brévignon, 2008 |
| *J. evarete evarete* Cramer, 1779 |
| *J. evarete occidentalis* C. & R. Felder, 1862 * |
| *J. evarete pallens* C. & R. Felder, 1867 |
| *J. flirtea* (Fabricius, 1793) * |
| *J. fuscencens* (Butler, 1901) * |
| *J. genoveva* (Cramer, 1780) |
| *J. genoveva constricta* C. & R. Felder, 1867 |
| *J. genoveva genoveva* (Cramer, 1780) * |
| *J. genoveva vivida* Forbes, [1929] |
| *J. grisea* Austin & Emmel, 1998 * |
| *J. hilaris* C. & R. Felder, [1867] * |
| *J. infuscata* C. & R. Felder, 1867 * |
| *J. litoralis* Brévignon, 2009 * |
| *J. neildi* Brévignon, 2004 * |
| *J. neildi varia* Grishin, 2020 |
| *J. nigralis* Forbes [1929] |
| *J. nigrosuffusa* Barnes & McDunnough, 1916 |
| *J. nigrosuffusa nigrosuffusa* Barnes & McDunnough, 1916 * |
| *J. nigrosuffusa stemosa* Grishin 2020 * |
| *J. oscura* Neild, 2008 |
| *J. pacoma* Grishin, 2020 * |
| *J. vestina* C. & R. Felder, 1867 |
| *J. vestina livia* Fruhstorfer, 1912 * |
| *J. vestina vestina* C. & R. Felder, 1867 * |

*J. wahlbergi* Brévignon, 2008 *
*J. zonalis* C. & R. Felder, 1867
*J. zonalis michaelisi* Fruhstorfer, 1907
*J. zonalis swifti* Brévignon, 2004
*J. zonalis zonalis* C. & R. Felder, 1867 *

**Old World *Junonia***
*J. adulatrix* Fruhstofer, 1903 *
*J. agnesberenyiae* Sáfián, 2018
*J. almana* Linnaeus, 1758 *
*J. ansorgi* Rothschild, 1899
*J. artaxia* Hewitson, 1864 *
*J. atlites* Linnaeus, 1763 *
*J. chorimene* Guérin-Méneville, [1844] *
*J. cymodoce* Cramer, [1777]
*J. cytora* Doubleday 1847 *
*J. erigone* Cramer, [1775] *
*J. goudoti* Boiduval, 1833 *
*J. gregorii* Butler [1896]
*J. hedonia* Linnaeus, 1764 *
*J. hierta* Fabricius, 1798
*J. hierta cebrene* Stoneham, 1965 *
*J. hierta hierta* Fabricius, 1798 *
*J. intermedia* C. & R. Felder, 1862 *
*J. iphita* Cramer, [1779] *
*J. lemonias* Linnaeus, 1758 *
*J. natalica* C. & W. Felder, 1862 *
*J. oenone* Linnaeus, 1758 *
*J. orithya* Linnaeus, 1758
*J. orithya here* Lang, 1884 *
*J. orithya madagascariensis* Guenée, 1865 *
*J. orithya orithya* Linnaeus, 1758 *
*J. rhadama* Boiduval, 1833 *
*J. sophia* Fabricius, 1793 *
*J. stygia* Aurivillius, 1894 *
*J. temora* Felder & Felder 1867 *
*J. terea* Crury, 1773 *
*J. timorensis* Wallace 1869
*J. touhilimasa* Vuillot, 1892 *
*J. villida* Fabricius, 1787 *
*J. westermanni* Westwood 1870 *

## Materials and Methods

**Specimen collection, preparation, and sequence generation.** A total of 97 specimens were included in the phylogenetic analyses, consisting of 64 *Junonia* (representing 40 of the 47 described species; Table 5-1), 21 additional samples from the tribe Junoniini (11 *Precis*, three *Hypolimnas*, two *Protogoniomorpha*, two *Salamis*, and three *Yoma*), and 11 more distantly related outgroup species within the subfamily Nymphalinae (one Victorinini, two Nymphalini, three Kallimini, two Melitaeini, one Coeini, one Kallimoidini, and one Doleschalliini; Electronic Supplement VI, https://doi.org/10.34990/FK2/I6QUPN/GVXXSH). Duplicate specimens for some species were used to ensure that mitochondrial diversity of as many different mitochondrial haplotype groups were represented, as well as to explore if reticulate evolution was also occurring in the Old World (as it does in the New World species), hybridization, and the possibility of cryptic species. Sequences in the data set were selected from samples acquired by the Marcus lab and sequence data for 10 specimens generated by other researcher groups were obtained from GenBank (KF590539; KT380025; KF199862; KF990127; KF590535; MG736927; CM002851; MK252271; SRX7439668; SRX7439671). Specimens were identified unambiguously in all cases to species based on a combination of specimen data (species descriptions and collection localities) and morphology (Electronic Supplement V, New World species only, https://doi.org/10.34990/FK2/I6QUPN/FO5GVH) and assigned a laboratory code. For sequences retrieved from GenBank from other research groups, species identification was also unambiguous in all cases based on specimen data such as species descriptions, and collection locality data provided in GenBank records and the papers that described these sequences (Electronic Supplement VI, https://doi.org/10.34990/FK2/I6QUPN/GVXXSH). DNA was extracted from a single butterfly leg per specimen with a Qiagen DNEasy Blood and Tissue Kit manually as previously described (Gemmell and Marcus 2015) or using a Qiagen QiAcube extraction robot (Qiagen, Düsseldorf, Germany) using the manufacturer's animal tissue DNA program. Then samples were stored at -20°C until they were sequenced.

Three methods were used to obtain sequence data. The first method involved two samples in a pilot project using Ion Torrent (ThermoFisher Scientific, Waltham, Massachusetts, USA) Next-generation sequencing technology at the Next-Generation Sequencing Platform facility at the Manitoba Institute of Child Health. The samples were sheared to an average fragment size of approximately 200 bp by sonication using a S220 Focused-Ultrasonicator (Corvaris, Woburn,

Massachusetts, USA). Evaluation of the fragments was done using a High Sensitivity DNA chip for the Bioanalyzer 2100 electrophoresis system (Agilent, Santa Clara, California, USA) using the standard manufacturer protocol. Then, 200 bp inserts were used to create a fragment library using the Ion PGM Template OT2 200 Kit and sequenced using the Ion PGM Sequencing 200 Kit v2 and either an Ion 314 Chip Kit v2 or an Ion 316™ Chip Kit v2 (ThermoFisher Scientific).

The second method was Illumina MiSeq (San Diego, California, USA) Next-generation sequencing where extracted DNA samples were processed at the Next-Generation Sequencing Platform facility at the Manitoba Institute of Child Health. Samples were sonicated and evaluated as described above except that they were sheared to an average fragment size of 600 bp. Indexed libraries were created from the sheared samples prepared for loading onto a MiSeq Next-generation Sequencing Instrument (Illumina) using A KAPA LTP Library Preparation Kit (Kapa Biosystems, Boston, Massachusetts, USA), and sequencing was accomplished using the MiSeq reagent V3 300X2 paired-end reagent kit (Illumina). The final method, Illumina NovaSeq6000 sequencing, was performed in a similar fashion to Illumina MiSeq at the Genome Quebec Next-generation sequencing facility at McGill University, except that the average fragment size was 300 bp, fragment libraries were prepared using the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, Massachusetts), and sequencing was accomplished using a NovaSeq 6000 S4 PE150 paired-end reagent kit (Illumina).

Paired-end sequencing was used preferentially because it yields high quality DNA sequences that allow researchers to align and easily detect insertion-deletion events, DNA rearrangements and repetitive DNA sequences, because the DNA is sequenced from both ends of a segment of DNA (Bashir *et al.* 2008). To generate the data sets used here, Geneious version 10.2.6 was used to assemble and annotate the newly generated sequences (mean size of each sequence library ~3.42 Gigabase pairs) to previously published complete mitochondrial genomes and to complete nuclear rRNA repeats generated within the Marcus lab (McCullagh and Marcus 2015; Peters and Marcus 2016; Peters and Marcus 2017; Lalonde and Marcus 2019b; Lalonde and Marcus 2019d; Alexiuk *et al.* 2020a; Alexiuk *et al.* 2020b; Hamilton *et al.* 2020; Lalonde and Marcus 2020c; Living Prairie Mitogenomics Consortium 2020; Payment *et al.* 2020a; Payment *et al.* 2020b; Lalonde 2021).

**Mitogenome Phylogeny.** Phylogenetic reconstruction was done using a total of 97 mitogenome sequences from 64 *Junonia* specimens from 40 species (36 currently unpublished, 25 published previously by the Marcus lab, 3 from Genbank), 21 specimens from other genera in the tribe Junoniini (11 *Precis* (10 unpublished, one published), three *Hypolimnas* (two unpublished, one obtained from GenBank), two *Protogoniomorpha* (one unpublished, one published), two *Salamis* (one currently unpublished, 1 published), three *Yoma* (two unpublished, one obtained from GenBank), and 12 specimens (one unpublished, 11 publicly available) as outgroups within the subfamily Nymphalinae: one Victorinini, two Nymphalini, three Kallimini, two Melitaeini, one Coeini, two Kallimoidini (one unpublished), and one Doleschalliini) (Electronic Supplement VI, https://doi.org/10.34990/FK2/I6QUPN/GVXXSH). Mitogenome sequences (Electronic supplement VII, https://doi.org/10.34990/FK2/I6QUPN/QUGYFO) were aligned in CLUSTALX 2.1 (Thompson *et al.* 1997; Larkin *et al.* 2007). Phylogenetic model selection was done using jModeltest 2.1.1 (Darriba *et al.* 2012) and analyzed using Bayesian Inference with the GTR+I+G model in MrBayes version 3.2.7 (Ronquist and Huelsenbeck 2003; Ronquist *et al.* 2012) with four chains (three hot and one cold) for 10 million MCMC iterations (convergence observed by average standard deviation of split frequencies below 0.01 (Ronquist and Huelsenback 2003)) along with sampling every 1000 generations, with the first 25% of iterations discarded as burn-in. The trees produced were viewed in FigTree version 1.4.3 (Rambaut 2016), rooted using the outgroup species *Baeotus beotus* (MW571038), and the final trees were generated using Canvas X Draw.

**Internal transcribed spacer regions and rRNA repeat phylogeny.** Phylogenetic reconstruction used a total of 90 nuclear rRNA repeats (containing rRNA and ITS sequences) comprised of 62 *Junonia* species (61 unpublished), 19 specimens from genera in the tribe Junoniini (11 *Precis* (10 unpublished), 2 *Hypolimnas* (both unpublished), 2 *Protogoniomorpha* (one unpublished), 2 *Salamis* (one unpublished), 2 *Yoma* (both unpublished)), and 9 specimens as outgroups (eight published) within the subfamily Nymphalinae (one Victorinini, one Nymphalini, three Kallimini, one Coeini, two Kallimoidini (one unpublished), and one Doleschalliini (Electronic Supplement VI, https://doi.org/10.34990/FK2/I6QUPN/GVXXSH). This analysis includes 7 fewer samples (two *Junonia*, two from the tribe Junoniini, and three outgroup species, Electronic Supplement VI, https://doi.org/10.34990/FK2/I6QUPN/GVXXSH) than the mitogenome data set because for those samples neither the assembled rRNA repeat

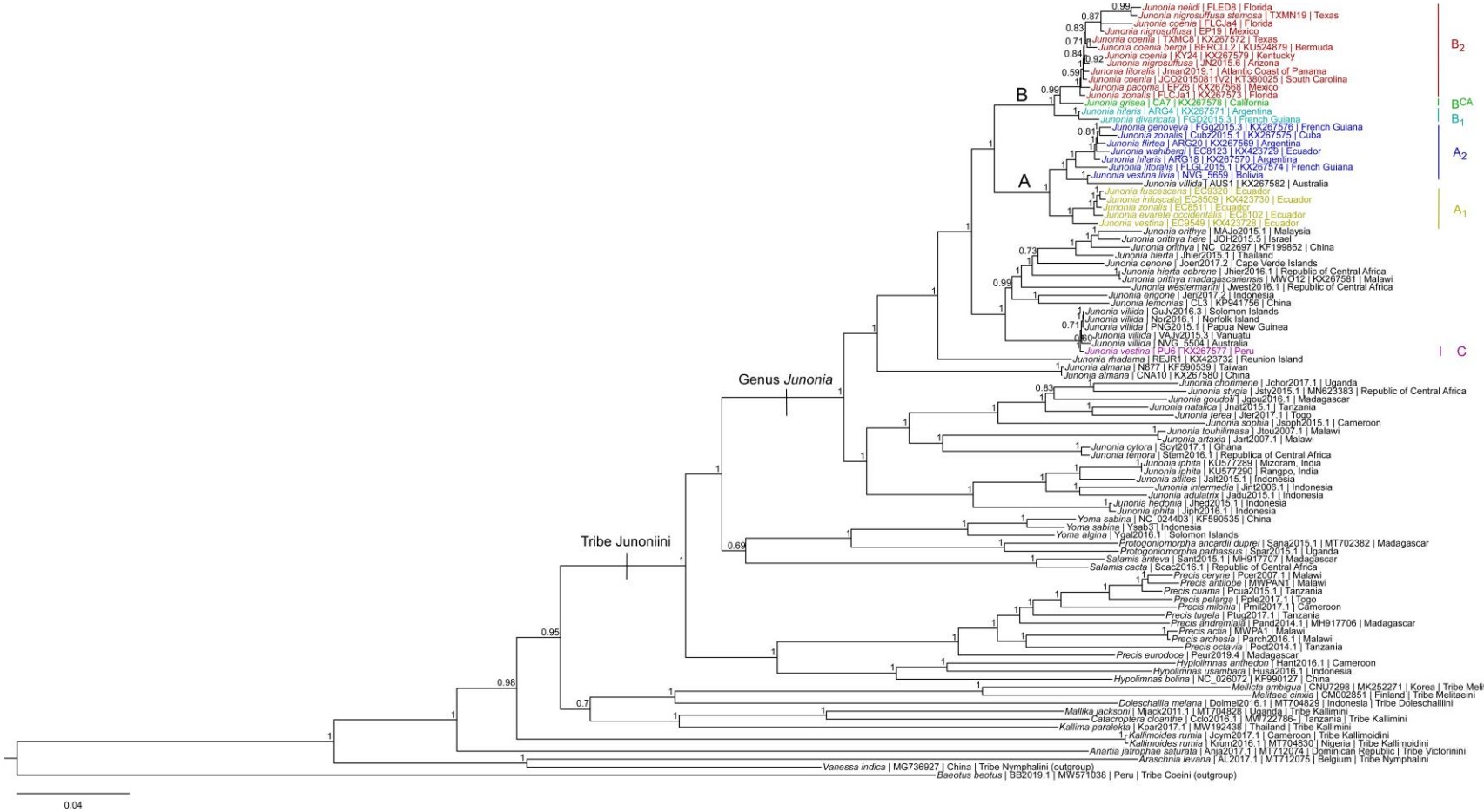
sequences, nor the SRAs containing raw sequence data were available from GenBank. Sequences were aligned (Electronic Supplement VIII, https://doi.org/10.34990/FK2/I6QUPN/6CBAJB)

in CLUSTALX 2.1 (Thompson *et al.* 1997; Larkin *et al.* 2007). Phylogenetic model selection was done using jModeltest 2.1.1 (Darriba *et al.* 2012) and analyzed using Bayesian Inference with the GTR+I+G model in MrBayes version 3.2.7 (Ronquist and Huelsenbeck 2003; Ronquist *et al.* 2012) with four chains (three hot and one cold) for 10 million MCMC iterations (convergence observed by average standard deviation of split frequencies below 0.01 (Ronquist and Huelsenback 2003)) along with sampling every 1000 generations, with the first 25% of iterations discarded as burn-in. The trees produced were viewed in FigTree version 1.4.3 (Rambaut 2016), rooted using the outgroup species *Baeotus beotus* (MW571038), and the final trees were generated using Canvas X Draw.

## Results

***Junonia* Mitogenome Phylogeny**. The mitogenome phylogeny was constructed using Bayesian inference with a GTR+I+G model with a best state likelihood of -170867 and a final average deviation of split frequencies of 0.002109 (Fig. 5-3). I obtained sequence data for 64 *Junonia* specimens (representing 40 of the 47 described species), 21 representatives of the five other genera in the tribe Junoniini, and 11 outgroup species within the family Nymphalinae. Based on these data the Tribe Junoniini resolves into a monophyletic clade. The New World species from genus *Junonia* are resolved into a single large clade with the exception of a single *J. villida* specimen from Australia grouping with the New World species and a single South American *J. vestina* specimen grouping with Old World taxa. The species-specific relationships in the New World are unresolved based on mitochondrial haplotypes (Fig. 5-3) and the individual species are not monophyletic in this analysis. Haplotype group A and haplotype subgroup $A_2$ remain the most prevalent in South American *Junonia* populations. Haplotype subgroup $A_{1,}$ originally thought to only occur in high elevation *J. vestina* populations, is also shared by some individuals of *J. fuscescens*, *J. infuscata*, *J. zonalis*, and *J. evarete* populations in Ecuador.

**Figure 5-3.** Bayesian Inference phylogeny (GTR+I+G model, best state likelihood = -170867 and a deviation of split frequencies = 0.002109) of the genus *Junonia* using mitochondrial DNA sequences of 64 *Junonia* species, 21 other species from the tribe Junoniini, and 12 outgroup species from other tribes within the subfamily Nymphalinae. Bayesian posterior probability values determined by MrBayes are given at each node. Haplotypes group assignments are displayed for New World specimens.



Haplotype group B is most prevalent in North America, Central America, and Bermuda. Haplotype subgroup $B_2$ is the most recently diverged within group B (Fig. 5-3) and is the predominant haplotype in the Western Hemisphere from Panama northwards. The $B^{CA}$ haplotype group from *J. grisea* and other *Junonia* in the American Southwest is the sister clade to the $B_2$ haplotype group in the New World. Haplotype $B_1$ seems to be the oldest B haplotype group and only occurs in South American *Junonia* populations, as the $B_1$ group diverged prior to the divergence of the $B_2/B^{CA}$ lineages.

Haplotype group C is restricted to *J. vestina* in Peru, and this haplotype group does not cluster with the other New World haplotype groups but is instead the sister clade to a group of mitogenomes from *J. villida* (an Old World species with an Indo-Pacific distribution). Similarly, a single *J. villida* mitogenome is the sister clade to the New World haplotype $A_2$ clade. The basal lineages of both haplotype groups A and B are of South American origin (Fig. 5-3). This suggests that there may be recent or historical gene flow between the New World *J. vestina* and Indo-Pacific populations of *J. villida*.

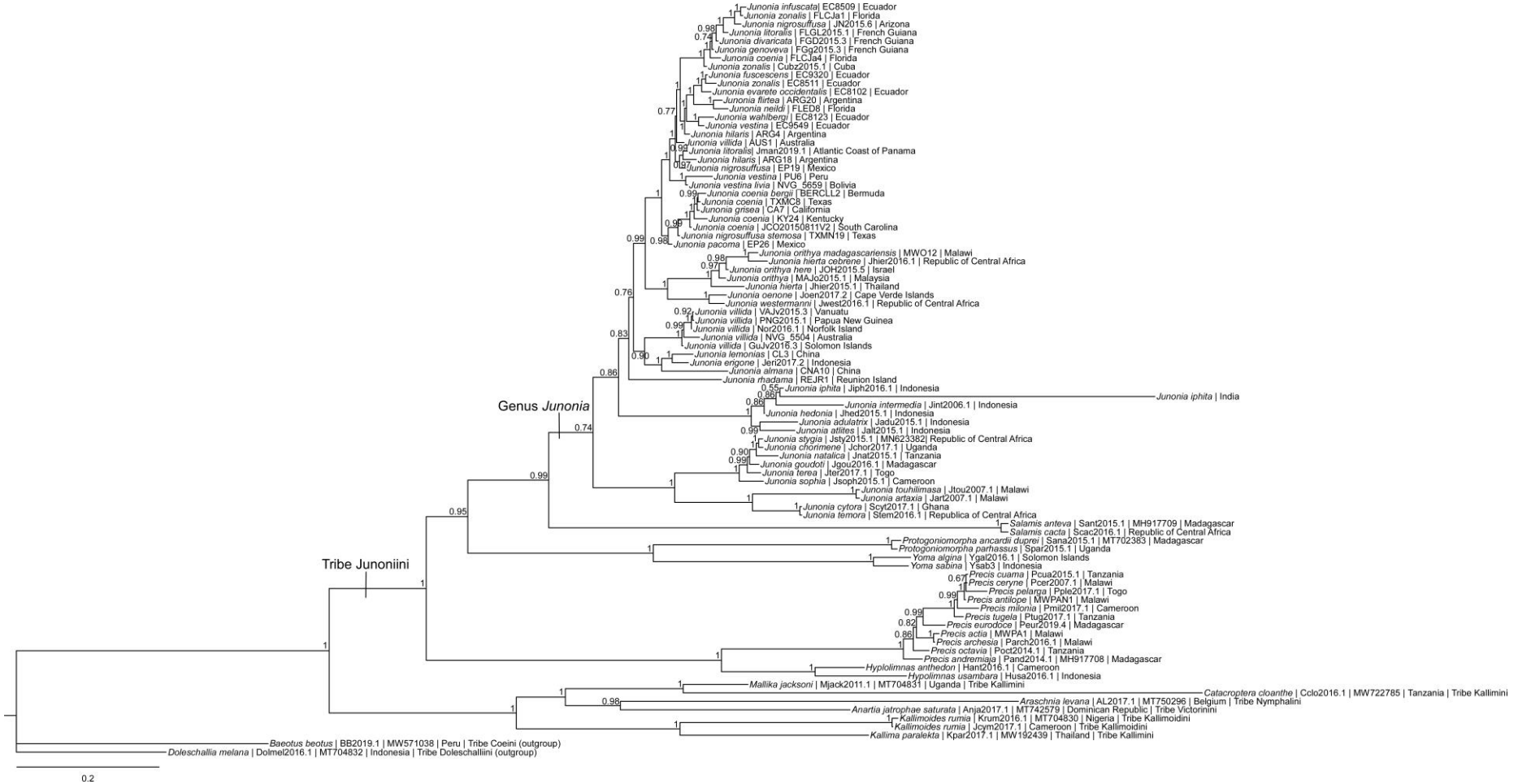
In contrast to the New World, prior studies had generally concluded that individual Old World *Junonia* species were monophyletic based on limited sampling of species and populations. The current analysis of complete mitochondrial genomes suggests that there is a lack of monophyly in at least some Old World *Junonia* species (Fig. 5-3). For example, in *J. orithya* and *J. hierta* there are two separate lineages, one in Asia and one in Africa, which are more closely related to the sympatric lineage of the other species than they are to their conspecific allopatric lineage. This suggests that there has either been remarkable parallel morphological and colour pattern evolution in these lineages or that there may be lateral transfer and introgression of mitochondrial haplotypes between them. Another species pair that may be experiencing introgression and ongoing geneflow are *J. iphita* and *J. hedonia*. The *J. iphita* mitogenome sequence from Indonesia forms a clade with sympatric sequences from *J. hedonia* rather than with *J. iphita* sequences from elsewhere in Asia. Collectively, these examples suggest that combinatorial (both nuclear and mitochondrial) genetic exchange through introgression is more widespread in *Junonia* than previously appreciated, including in the Old World.

Phylogenetic analysis of mitochondrial genome sequences shows that tribe Junoniini forms a monophyletic group (Fig. 5-3). The basal group among the mitogenomes of the Junoniini was a clade containing *Precis* and *Hypolimnas* species and a Bayesian posterior probability of 1

(Fig. 5-3), a finding that is consistent with previous phylogenetic reconstructions (Kodandaramaiah and Wahlberg 2007; Clarke 2017; Pyrcz *et al.* 2021). The sister clade to *Junonia* in the mitogenome tree is a clade containing genera *Protogoniomorpha*, *Yoma*, and *Salamis*, with *Salamis* being the basal taxon in the clade, but the Bayesian posterior probability value supporting this node is low (0.69), suggesting that the placement of the genus *Salamis* may need to be re-evaluated when more data become available.

***Junonia* internal transcribed spacer regions (ITS) and rRNA repeat phylogeny.** The nuclear rRNA repeat phylogeny was constructed for 90 specimens from the subfamily Nymphalinae using Bayesian inference with a best state likelihood of -119000 and a final average deviation of split frequencies of 0.003566 (Fig. 5-4). I obtained sequence data for 62 *Junonia* specimens (representing 40 of the 47 described species), 19 representatives of the five additional genera found within the tribe Junoniini, and nine outgroup species from within the family Nymphalinae. For the genus *Junonia* all sequence data (except for a single species; *J. stygia* where the nuclear rRNA repeat was previously published by the Marcus lab (Living Prairie Mitogenomics Consortium 2020)) is released and analyzed here for the first time. All nuclear rRNA repeat sequences from the other five genera in the tribe Junoniini are also newly generated data produced for this project and all are unpublished except for three sequences that I published as part of my dissertation work (Lalonde and Marcus 2019b; Lalonde and Marcus 2019d; Lalonde and Marcus 2020c). All outgroup sequence data was generated and published by the Marcus lab (Lalonde and Marcus 2019b; Lalonde and Marcus 2019d; Alexiuk *et al.* 2020a; Alexiuk *et al.* 2020b; Hamilton *et al.* 2020; Lalonde and Marcus 2020c; Payment *et al.* 2020a; Payment *et al.* 2020b; Lalonde 2021; Lalonde 2022).

**Figure 5-4**. Bayesian Inference phylogeny (GTR+I+G model, best state likelihood = -119000 and an average deviation of split frequencies = 0.003566) of the genus Junonia using complete rRNA repeat sequences from 62 Junonia specimens, 19 other species from the tribe Junoniini, and 9 outgroup species from other tribes within the subfamily Nymphalinae. The Bayesian posterior probability values determined by MrBayes are given at each node.



Based on nuclear rRNA repeat sequences, the Tribe Junoniini as a whole and each of the genera within it resolve into a monophyletic clade similar to what was found using mitogenome data (Fig. 5-4), although the support for the genus *Junonia* is lower (0.74). Also similar to the mitogenome phylogeny, New World *Junonia* species relationships do not resolve into monophyletic taxa. Instead, the nuclear rRNA repeats of the New World *Junonia* form two distinct clades. The members of one clade appear to be restricted to specimens from North America and Bermuda. The sequences making up the other clade originate from South America, Central America, the Caribbean, and the southern parts of North America. Some New World *Junonia* species include individuals assigned to both clades, which is not surprising as hybridization and introgression is ongoing between the many species in the New World (Hafernik 1982; Lalonde and Marcus 2019c; Cong *et al.* 2020). As a whole, the New World *Junonia* are monophyletic with only a single exception; the same Old World specimen of *J. villida* that grouped with the New World in the mitogenome phylogeny is also clustering with nuclear rRNA repeats from the New World. The *J. vestina* specimen with a distinct mitogenome (haplotype group C) that had grouped with the Old World *J. villida* clade in the previous analysis (Fig. 5-3), is the sister taxon to another South American *J. vestina* sample within the New World clade in the nuclear rRNA repeat phylogeny (Fig. 5-4).

The nuclear rRNA repeat phylogeny for the Old World *Junonia* was consistent with the mitogenome phylogeny with two major differences. The *J. villida* clade, for which a *J. vestina* sequence from the New World was the basal lineage in the mitogenome tree, is more closely associated with a group of Asian *Junonia* species (*J. lemonias*, *J. erigone*, *J. almana*) in the nuclear rRNA repeat phylogeny. The second difference is that the sister clade to this Asian clade (*J. lemonias*, *J. erigone*, *J. almana*) in the mitogenome tree, is the sister clade to the New World *Junonia* in the rRNA repeat phylogeny (Fig. 5-4). This clade, which includes both African and Asian lineages, contains *J. orithya* and *J. hierta.* The African lineages of *J. orithya* and *J. hierta* form a clade together and Asian lineages from these two species are interspersed with one another, which shows similarities to the results of the mitogenome analysis. In contrast, the nuclear rRNA repeats from *J. iphita* form a monophyletic group and do not show the close affinity with *J. hedonia* sequences as observed in the mitogenome phylogeny (Fig. 5-3).

The relationships within the Tribe Junoniini based on the rRNA repeat data set are consistent with the mitogenome analysis except for the placement of the genus *Salamis*, which is

placed as the sister taxon to *Junonia* with a very high probability value (0.99). This gives further support to the African origin of genus *Junonia* (Kodandaramaiah 2009; Pfeiler *et al.* 2012) as the genus *Salamis* is restricted to Africa. The relationships of the outgroup species are consistent with the mitogenome analysis, with the exception of the tribe Kallimini. Based on the nuclear rRNA repeat analysis, the tribe Kallimini is paraphyletic, which was not observed in the mitogenome phylogeny. Two of the genera that are assigned to tribe Kallimini (*Mallika jacksoni* and *Catacroptera cloanthe*) group as expected, but the third representative of the Kallimini, *Kallima paralekta*, clusters with tribe Kallimoidini within subfamily Nymphalinae (Fig. 5-4).

## Discussion

**Tribe Junoniini, the sister taxon to the genus *Junonia*, and the paraphyly of Kallimini.**
The sister lineages within the Junoniini (Fig. 5-3, Fig. 5-4), include representatives from a clade containing both *Precis* and *Hypolimnas* taxa, both themselves monophyletic which is consistent with previous studies (Kodandaramaiah and Wahlberg 2007; Clarke 2017; Pyrcz *et al.* 2021). The sister clade to *Junonia* differs between my phylogenetic analyses. Based on the mitogenome phylogeny, it contains the genera *Protogoniomorpha*, *Yoma*, and *Salamis*, with *Salamis* being the basal taxon in the clade that is consistent with previous analyses in the group (Kodandaramaiah and Wahlberg 2007; Clarke 2017; Lalonde and Marcus 2020c; Pyrcz *et al.* 2021). Although Pyrcz et al. (2021) found the genus *Salamis* to be contained among the basal branches of genus *Junonia*. The rRNA repeat data show *Salamis* to be the sister clade to the genus *Junonia* with high support (posterior probability value of 0.99), with the next further outgroup to *Junonia* containing the genera *Yoma* and *Protogoniomorpha*. This finding adds additional support to previous studies which also found *Junonia* to have African origins, as the genus *Salamis* is restricted in their distribution to Africa, as are the earliest diverging lineages within *Junonia*. The distribution of *Salamis*, their usual basal placement in the clade containing *Yoma* and *Hypolimnas*, and the high node support value gives great support to the hypothesis that this genus sister taxa to the genus *Junonia* is correct.

The genus *Kallima* (Tribe Kallimini) was originally a catch-all genus for many Asian and African nymphalid butterflies masquerading as leaf mimics (Skelhorn 2015). It was later found that based on morphological differences in genitalia and behaviour that the genus *Kallima* is

actually restricted to Asia, and all African leaf mimicking species that were originally assigned to this genus were reassigned one of three other genera within Nymphalinae (*Junonia* (Tribe Junoniini), *Mallika* (Tribe Kallimini), or *Kallimoides* (Tribe Kallimoidini)) (Shirôzu and Nakanishi 1984; Larsen 1999; Wahlberg *et al.* 2005). The Tribe Kallimini was considered to be comprised of the genera *Kallima*, *Mallika*, and *Catacroptera* and viewed as the sister clade to the Junoniini, since most of the Kallimini and the basal lineages of the Junoniini are all leaf mimics (Larsen 1999). Molecular phylogenetics has consistently placed tribe Melitaeini as the sister to the Junoniini, and places tribe Kallimini more basally (Wahlberg *et al.* 2005; Su *et al.* 2017). While the mitogenome tree (Fig. 5-3) is consistent with these results, phylogenetic reconstruction based on the rRNA repeat region (Fig. 5-4) shows genera *Mallika* and *Catacroptera* (both African genera) forming a monophyletic clade. Instead, the genus *Kallima* (*Kallima paralekta*) falls outside of this grouping and is the sister taxon to genus *Kallimoides*, with a Bayesian probability value of 1 (Fig. 5-4).

This difference in the monophyly of Kallimini may be attributable, at least in part, to the limited taxon sampling among the outgroups within the rRNA repeat phylogeny (Fig. 5-4). Both the Junoniini and the Kallimini show close molecular phylogenetic associations with the Melitaeini in our mitogenome trees (Fig. 5-3, Fig. 5-4) and in previous studies (Wahlberg *et al.* 2003; Freitas and Brown 2004; Wahlberg *et al.* 2005) but the nuclear rRNA repeat sequences are not yet available from representatives of the Melitaeini. Although not the primary focus of this study, additional taxon sampling in the Kallimini and Melitaeini for nuclear rRNA repeats would be helpful for inferring higher level taxonomic relationships within the Nymphalinae,

***Junonia*.** The current study, using both the full mitochondrial genome and nuclear rRNA repeat regions from both Old and New World taxa, is the most comprehensive phylogeny of *Junonia* ever assembled (Fig. 5-3, Fig. 5-4). Based on the mitogenome and rRNA repeat reconstructions of the *Junonia* butterflies, the placement of the Old World *Junonia* taxa are consistent with previous studies (Wahlberg *et al.* 2005; Kodandaramaiah and Wahlberg 2007; Kodandaramaiah 2009; Clarke 2017; Pyrcz *et al.* 2021). Previous Old World *Junonia* phylogenies included 19 of the 29 described species (Kodandaramaiah and Wahlberg 2007; Kodandaramaiah 2009; Clarke 2017; Pyrcz *et al.* 2021) but considered a maximum of three New World taxa. More recent New World *Junonia* DNA barcoding studies have included 19 of 29 Old World species (Borchers and

Marcus 2014; Gemmell *et al.* 2014; Gemmell and Marcus 2015), and mitogenome phylogenetic studies have included between 8 and 11 New World species (Peters and Marcus 2017; Cong *et al.* 2020; Lalonde and Marcus 2020c). This enhanced coverage of the entire genus, with the addition of additional taxa from within the Tribe Junoniini makes the accurate interpretation of taxonomic relationships and evolutionary patterns within *Junonia* possible.

**New World *Junonia.*** As expected, the full mitochondrial genome dataset was not able to resolve the species level relationships in the New World (Fig. 5-3) and the same patterns associated with lack of monophyly in DNA barcode phylogenies found in previous studies remains apparent (Fig.1-1, Fig. 5-1, (Pfeiler *et al.* 2012; Borchers and Marcus 2014; Gemmell *et al.* 2014; Gemmell and Marcus 2015; Peters and Marcus 2017; Cong *et al.* 2020)). Many New World species include individuals that carry both A and B haplotype groups. This is reinforced by the nuclear rRNA repeat phylogeny that shows a similar pattern where most New World *Junonia* species do not form monophyletic groups. This is indicative of current gene flow between the New World *Junonia* species and is consistent with prior observations of hybridization between many of these species (Hafernik 1982; Lalonde and Marcus 2019c; Cong *et al.* 2020; Lalonde and Marcus 2020a). Based on the mitogenome phylogeny (Fig. 5-3) the New World *Junonia* is monophyletic with two exceptions. The first is a *J. vestina* sample that possesses haplotype group C and is most closely related to a lineage of *J. villida* from the Indo-Pacific. This is somewhat different than the findings of Peters and Marcus (2017), which placed *J. vestina* as the sister taxon to *J. lemonias*, but that study included only single examples of most *Junonia* species, including *J. villida*, so this discrepancy can be attributed to the limited sampling of the earlier study. Both in Peters and Marcus (2017) and in the current study, there is a single *J. villida* sample from Australia that seems to be most closely related to the haplotype group $A_2$ in the New World. These findings signify that gene flow across the Pacific may be occurring and is of importance to understanding the relationships between the Old World and New World species, and of the origins of *Junonia* in the New World.

Complete nuclear rRNA repeats (~8,000 bp) occurs at high copy number and are easily recovered from the same genome skimming data sets used for assembling whole mitogenomes (Fig. 5-4, (Marcus 2018)). Phylogenetic analysis of *Junonia* nuclear rRNA repeats did not recover the same clades found through analyses of mitogenomes, nor do rRNA repeats resolve

the species level relationships within the New World. Many species were not monophyletic in the rRNA repeat analysis, nor was there any apparent correlation with life history traits such as host plant use (Electronic Supplement V, https://doi.org/10.34990/FK2/I6QUPN/FO5GVH). There does seem to be a geographic signal, with most North American specimens forming one rRNA repeat lineage, while a second lineage is made up of South American, Caribbean, and a few specimens (one Mexican *J. nigrosuffusa* and one Florida specimen each from *J. neildi*, *J. coenia*, and *J. zonalis*) from southern portions of North America. Based on the nuclear rRNA repeat data set, the New World *Junonia* are monophyletic, except for an Australian *J. villida* sample that groups with the New World in the mitogenome phylogeny. Unlike the mitogenome phylogeny, the nuclear rRNA repeats of all *J. vestina* samples fall within the New World rRNA clade. This further supports that hypothesis that gene flow between New World *J. vestina* and the Indo-Pacific *J. villida* may be ongoing since individual specimens from these species do not share the same level of molecular affinity with one another, with some specimens forming clades with the other taxon. I am also aware of one specimen of *J. villida* reportedly collected from the vicinity of Ajoya, Sinaloa, Mexico by Rudolf Hans Bock between 1972 and 1975 (California Academy of Sciences Collection), which may provide further support for the current gene flow hypothesis. I have examined the specimen and it is identified correctly as *J. villida*, but it is also possible that this specimen is mislabeled with incorrect collection locality data.

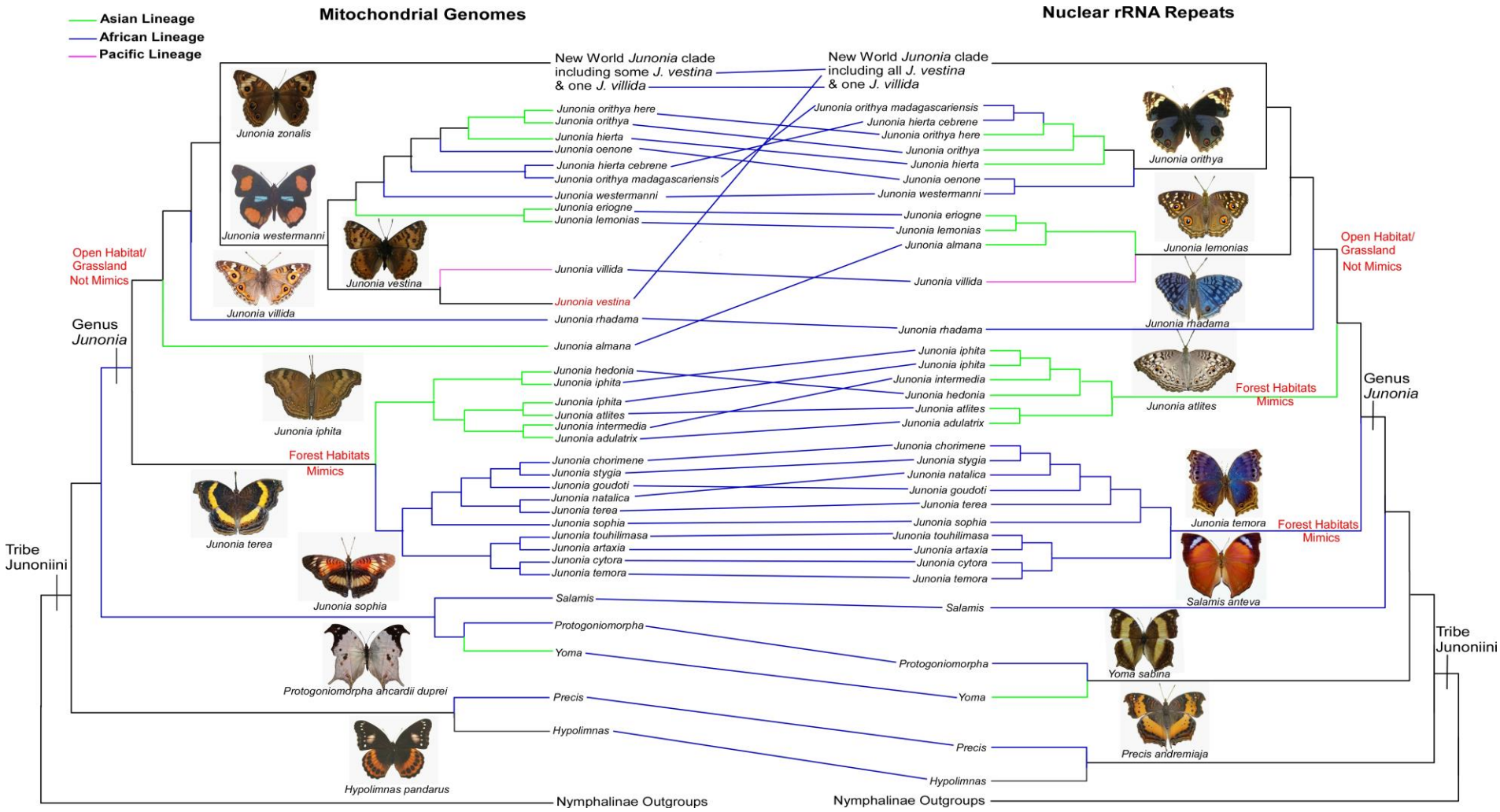
**Old World *Junonia.*** Most previous phylogenetic studies of Old World *Junonia* were unable test for monophyly or make strong statements about species level relationships, because only a single sample of each species was used (Wahlberg *et al.* 2005; Kodandaramaiah 2009; Borchers and Marcus 2014; Gemmell *et al.* 2014; Gemmell and Marcus 2015; Clarke 2017; Lalonde and Marcus 2020c; Pyrcz *et al.* 2021). The exception was Kodandaramaiah and Wahlberg (2007), which included multiple specimens from some Old World species that are comparable with this analysis. I will focus on two interesting lineages. First, specimens from Asian and African populations of *J. hierta* and *J. orithya* form clades based on geography rather than taxonomic species assignment. This was also apparent in some figures in Kodandaramaiah and Wahlberg (2007), but those authors did not comment on the pattern in the text of their paper. We have recovered the same pattern in both the phylogenetic analyses of mitogenome (Fig. 5-3) and nuclear rRNA repeat (Fig. 5-4) data sets. This clade is also the sister clade to the New World

*Junonia* in the analyses of Kodandaramaiah and Wahlberg (2007). The mitogenome data also places these taxa in the sister clade to the New World *Junonia,* but also includes *J. villida* as the basal group within the clade.

The second interesting observation was the phylogenetic association of Asian species *J. iphita* and *J. hedonia*. Consistent with the previous data available (Kodandaramaiah and Wahlberg 2007), mitogenomes from *J. iphita* specimens pair with either *J. hedonia* or *J. atlites* (Fig. 5-3). In contrast, in the analysis of rRNA nuclear repeats (Fig. 5-4), the *J. iphita* sequences form a monophyletic clade and none are sister to *J. hedonia*. A recent lateral transfer of the *J. hedonia* mitogenome to *J. iphita* in Indonesia seems likely as the sequences are nearly identical, but additional sampling and sequencing from these and other species in this lineage would clarify patterns of organelle capture in Old World *Junonia.* What seems increasingly clear is that while lateral transfer events may be most frequent in the New World *Junonia*, it also occurs in some Old World lineages in this genus and may have contributed to reticulate evolution of the genus as a whole.

Despite observed instances of apparent lateral transfer, the Old World *Junonia* form distinct molecular clades made up of species that share many phenotypic features (Fig. 5-5). Although there are small differences between the two different phylogenetic analyses in respect to species placement, all Old World species group based on habitat type, geography, and mimicry strategy. The basal *Junonia* clades in the mitogenome phylogeny (Fig. 5-5) consist entirely of forest dwelling species. Within this group there are two main lineages. The first is an Asian forest-dwelling butterfly lineage containing species (*J. iphita, J. atlites*, *J. intermedia, J. adulatrix*, *J. hedonia*) that have adults that at rest with wings closed masquerade as leaves (Skelhorn 2015), although somewhat imprecisely. Most of these species have brown or grey wings dorsally. The second lineage includes African species that are again all forest dwelling but most of these species are considered to be very good leaf mimics (Fig. 5-5; Clarke 2017). There is one exception, *J. sophia,* which is thought to be a Batesian mimic of the false diadem butterfly (*Pseudacraea lucretia*) (Larsen 1999). However, *Pseudacraea lucretia* mimics species in the genus *Acraea*, so *J. sophia* may also be a mimic of *Acrea* species that occur in the same habitat. Within this African *Junonia* lineage, there are 2 subclades, one in which all the species are iridescent blue on their dorsal wing surfaces, and one in which the dorsal wing surfaces are either dark brown or dark brown and yellow.

**Figure 5-5.** Modified molecular phylogeny of the Old World *Junonia*. Interpretations are based on mitogenome and rRNA repeat phylogenetic reconstructions from figures 2 and 3. Geographic origins, habitat preferences, and the display of mimicry of lineages are indicated.



The next large *Junonia* clade recovered by analysis of mitogenomes has transitioned away from forest habitats. It includes all of the New World *Junonia* species as well as some Asian (*J. orithya, J. hierta, J. lemonias, J. villida, J. erigone, J. almana*) and African species (*J. orithya, J. hierta, J. oenone, J. westermanni, J. rhadama*), which are considered to be grassland or open habitat specialists, and none are considered to be mimics of any kind (Fig. 5-5; Aoki *et al.* 1982; Larsen 1999). Consistent with prior studies, this lineage originated in Africa, dispersed to Asia, and then returned to Africa, perhaps several times (Kodandaramaiah and Wahlberg 2007). Later, as will be discussed below, this lineage was responsible for establishing the New World *Junonia* radiation.

The phylogenetic reconstruction based on the nuclear rRNA repeats yields similar results to the mitogenome analysis, with phylogenetic trends based on habitat type, mimicry, and geography, but differences do exist. The basal lineage of *Junonia* in this analysis supports an African origin for this genus, as all species that occur in this clade are forest-dwelling and restricted to Africa (Fig. 5-5) reinforcing phylogenetic results based on mitogenomes and an earlier 3-gene analysis (Fig. 5-4; Kodandaramaiah and Wahlberg 2007). Like the mitogenome phylogeny, all species in this basal clade are mimics (either masquerade mimics of leaves or Batesian mimics of aposematic distasteful butterfly species) but not all mimics in the dataset are included in this clade. The next clade to branch off the tree consists of mostly Asian and Indo-Pacific *Junonia* species, with the exception of *J. rhadama* that occurs in Madagascar and several Indian Ocean islands. The first major split in this clade is by habitat type: there are the forest dwelling imperfect leaf mimics, and the grassland and open habitat species.

The forest dwelling species include the *J. iphita* and *J. hedonia* complex discussed above in the context of the mitogenome phylogeny and the same pattern is seen here. The open habitat species include *J. rhadama* as the basal taxon. The remaining open habitat species are more morphologically similar to the New World than they are to other Old World Species. There is a key difference in the composition of this clade between the mitogenome and nuclear rRNA repeat phylogenetic results. In the mitogenome analysis, *J. villida* is the basal lineage in the sister clade to the New World *Junonia* (Fig. 5-5), while in the nuclear rRNA repeat analysis the sister clade is limited to African *J. westermanni*, and *J. oenone* and African/Asian *J. orithya* and *J. hierta*, while the remaining taxa in this clade (Asian: *J. villida, J. lemonias, J. erigone,* and *J. almana*) are transferred into a separate, slightly more basal lineage (Fig. 5-5). This difference

between the mitogenomic and nuclear rRNA repeat phylogenies raises the intriguing possibility that the New World *Junonia* were established with contributions from two Old World *Junonia* lineages: the lineage containing *J. villida* which crossed the Pacific Ocean, and the lineage containing *J. orithya* and *J. hierta*, which could have crossed either the Atlantic or Pacific Ocean to reach the New World. Thus, several of the early hypotheses for the origin of the New World *Junonia* may not have been mutually exclusive and instead may turn out to be simultaneously correct (Eliot 1946; Eliot 1947; Forbes 1947).

## Conclusions

Species delimitation among the New World *Junonia* was not possible with either complete mitochondrial genomes or nuclear rRNA repeats. Species delimitation using these sequences was more successful among Old World *Junonia* taxa, even though lateral transfer appears to have taken place in some Old World lineages as well. This may be a function of the apparent higher frequency of hybridization and the greater extent of reticulate evolution among the New World species. The large number of New World *Junonia* (18 species) that have evolved in a very short period of time (2-4 million years) suggest that speciation in this group has been accelerated compared to the older lineages in the Old World *Junonia* (28 species) which diverged since the origins of the genus 15-27 million years ago (Kodandaramaiah and Wahlberg 2007). Lateral transfer of adaptive genes and traits and reticulate evolution may have contributed to the substantially greater rate of speciation in the New World, making this system similar to the explosive species radiation events such as the African Rift Lake cichlid fish (Keller *et al.* 2013) or the Hawaiian Drosophila (Edwards *et al.* 2007; Price and Muir 2008).

To address the challenges of species delimitation in *Junonia*, one recent study successfully employed complete Z chromosome sequences (Cong *et al.* 2020). Although their data set only included specimens from some New World *Junonia* species, they were able to resolve the species into monophyletic clades. Accumulating complete Z chromosome sequences from the rest of the genus will provide an interesting comparison to results from analyses of mitochondrial genomes and nuclear rRNA repeats and together this may permit better delimitation of the remaining species, further resolve species level phylogenetic relationships, and should be considered for future phylogenetic studies of this genus.

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# Chapter 6: Discussion and Future Directions

The buckeye butterflies (genus *Junonia*) are considered an important model system for evolution and developmental biology. Traditionally the goal in developmental biology has been to find organisms with interesting features and that are relatively easy to grow, so that researchers can attempt to understand how these features develop. This was the primary focus for much of the developmental biology work in buckeye butterflies as they have features (specifically eyespots) that are relatively easy to manipulate (Nijhout 1980a; Nijhout 1980b; Nijhout 1984; Nijhout 1985; Nijhout 1986). Experimental work on this genus started in the early 1980's and focused primarily on the developmental biology of New World species, specifically looking at eyespots (Nijhout 1980a; Nijhout and Grunert 1988) and colour pattern development (Nijhout 1980b; Nijhout 1984; Koch and Nijhout 1990). Other work examined the effects of chemical ecology on larval growth and oviposition preference (Bowers and Puttick 1986; Pereyra and Bowers 1988; Fajer *et al.* 1991), host plant specificity (Bowers 1984; Stamp and Bowers 1992; Klockars *et al.* 1993), and the effects of predation (Stamp and Bowers 1991; Dyer and Bowers 1996; Bowers and Stamp 1997). Both of these fascinating research programs continue (Knerl and Bowers 2013; Nijhout *et al.* 2018; Carper *et al.* 2019; McKenna and Nijhout 2021), and additional interest has come from these topics from other research groups (Sekimura *et al.* 2015; Dhungel *et al.* 2016; Iwata and Otaki 2016), but until very recently, there been very few studies that compared both traits and these biological processes among *Junonia* species. Most work focused on a single species, *Junonia coenia*, and little attempt was made to associate these phenotypes and developmental processes with the evolutionary history of the group.

As the experimental work described above was occurring, other researchers focused on the taxonomy on the group although the literature is somewhat sparse with only a handful of species considered to occupy the New World (Hafernik 1982; Turner and Parnell 1985; Harvey 1985). Prior to 2008 only four *Junonia* species were thought to occur in the New World (Brévignon 2004; Neild 2008), and only one species (*Junonia coenia*) was thought to occur in North America, where the important developmental and chemical ecology work was being conducted. In contrast, taxonomic work in the twenty-first century suggests that there are at least 18 New World Junonia species (Brévignon 2004; Brévignon 2009; Brévignon and Brévignon 2012; Gemmell and Marcus 2015; Lalonde and Marcus 2019b; Cong *et al.* 2020), with at least seven species occurring in North America (Lalonde and Marcus 2019b). In some cases, the geographic localities from which specimens were collected for early experiments were home to

populations of more than one *Junonia* species. This sometimes poses a problem in recognizing which species were used in some of the early experiments because individuals from multiple species may have been inadvertently incorporated in some of these studies.

The main goal in evolutionary biology is to understand the evolutionary relationships, and making connections to how processes, features, and life history traits have evolved. Although the Marcus lab had been working in this area for several years prior to when I started my work, no researchers working on New World species had made connections between the evo-devo of butterfly colour patterns with the evolutionary history of genus *Junonia*, nor were they possible at the time. Some studies had examined Old World species, looking at the diversification of the group (Kodandaramaiah and Wahlberg 2007) and evolution of eyespots (Kodandaramaiah 2009) using morphology and short DNA sequence fragments. Only three New World species were considered in these studies, two of which may not have been identified correctly due to inconsistent and conflicting taxonomic designations in use at the time. Early systematic studies (prior to 2014) attempted to use short fragments of DNA to delimit New World species and reconstruct their evolutionary relationships, but due to inconsistent taxonomic designations, cryptic species, and the limited information contained in the DNA fragments used, delimiting species with this approach failed (Wahlberg *et al.* 2005; Pfeiler *et al.* 2012).

Since 2014, the Marcus lab has attempted to reconsider all of the findings from this evo-devo model system in the context of the evolutionary history of *Junonia* butterflies (Borchers and Marcus 2014; Gemmell *et al.* 2014; Gemmell and Marcus 2015; McCullagh 2016; Peters and Marcus 2017; Lalonde *et al.* 2018; Lalonde and Marcus 2019a; Lalonde and Marcus 2019b). Prior to my dissertation, some effort had been made to associate taxonomic designations with life history data of each species in North America, Mexico, and the Caribbean. This allowed for the use of biogeographic patterns to predict potential hybrid zones, glacial refugia, potential routes of colonization into the New World, and how these species may have diversified after glaciation periods (McCullagh 2016; Peters and Marcus 2017; Lalonde *et al.* 2018; Lalonde and Marcus 2019a; Lalonde and Marcus 2019b). My PhD work adds to this understanding by addressing additional questions relating to the invasion history of this group in the New World (Chapter 3 and 4), characterizing all New World species (Chapter 4), and answering the long-standing question of whether the genus *Junonia* is a ring species (Chapter 4). I was able to validate a very

useful high throughput method of mitochondrial haplotyping, that allows interpretation of gene flow between morphologically distinct species (Chapter 2) and used this method to map gene flow and demonstrate that extensive sharing of mitogenomes is still occurring. Observing gene flow in areas where it was thought that there were discontinuities in gene flow (Chapter 4) allowed me to falsify the *Junonia* ring-species hypothesis. I have also created the most comprehensive molecular-based phylogenies to date, that can be used to infer phylogenetic relationships (Chapter 5) among both the Old and New World *Junonia* species. With these phylogenetic reconstructions I have interpreted perceived relationships among *Junonia* taxa based on morphology and life history data and have provided additional support for a possible trans-Pacific route for the *Junonia* species to occupy the New World (Chapter 5).

As the buckeye butterflies (genus *Junonia*) are considered an important model system for evolution and developmental biology, I have been able to fill in the evolutionary context that has been lacking. Using Next-generation sequencing, I have added considerable amounts of Next-generation sequence data, with the additions of 36 new mitogenome sequence assemblies from the genus *Junonia*, as well as 19 new mitogenome sequences representing five additional genera from the tribe Junoniini. I have also added 61 new nuclear rRNA repeat sequences for the genus *Junonia*, plus 19 new nuclear rRNA repeats sequences representing the other 5 genera within the tribe Junoniini (Chapter 5). All data will be made publicly available for future research in fully assembled form so that they can be used as reference sequences, and the raw data will also be made available as Sequence Read Archives (SRAs) so that future studies may explore other pieces of the *Junonia* genome across many species in the genus. I have been able to make associations with taxonomy and life history data to provide a universal tool for identification of all New World *Junonia* species which will help guide future research, so that more comprehensive comparisons can be made between species (Chapter 4). It will also provide guidance on the interpretation of previous work where specific species designations are not clear.

## Future Directions

The genus *Junonia* is already a valuable model for many fields of experimental research and the new tools provided in this dissertation will enhance it even further. More work must continue to ensure that all species are characterized by Next-generation sequencing to aid in

comparative biology experimentation. There are at least three New World species and 10 subspecies missing from my analysis from South America, and five Old World species with many subspecies that need to be explored further. In addition, to characterize the variation that occurs within species groups in the genus, additional representatives from each species should be sampled to help clarify future taxonomic questions and help clarify the species level relationships in the New World. To address the challenges of species delimitation in *Junonia* using molecular data, large nuclear DNA molecules, such as the Z chromosome, have provided some promising early results with a handful of New World species (Cong *et al.* 2020).If studies of Z chromosome sequences could be extended to Old World and the remaining unstudied New World Species, it may provide a fuller phylogenetic understanding of the evolutionary history of this genus, particularly in comparison with existing phylogenetic reconstructions using mitochondrial genomes and nuclear rRNA repeats. Using a combination of sequences together, representing maternal and bi-parental lineages, may permit better delimitation of the remaining species, further resolve species level phylogenetic relationships, and permit important insights into the evolutionary history of this genus, so this strategy should be considered for future phylogenetic studies of this genus.

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**Appendix I: Hemisphere-wide New World *Junonia* range maps.** Lalonde, 2021. Chapter 4. The Chronicles of Junonia: Testing the ring species hypothesis in the New World buckeye butterflies. PhD Thesis "New World diversification and origins of the Buckeye butterflies (genus Junonia, Nymphalidae: Nymphalini)". Supervised by Jeffrey Marcus.

**Figure 1**. Whole geographic study area species distribution and proportion haplotype A for *Junonia divaricata* (86 samples). Proportion of haplotype A is represented using a colour gradient with red being 100% group A and purple being 0% group A. Species designations are represented using different shapes and the population size is represented by the size of the shapes on the map.

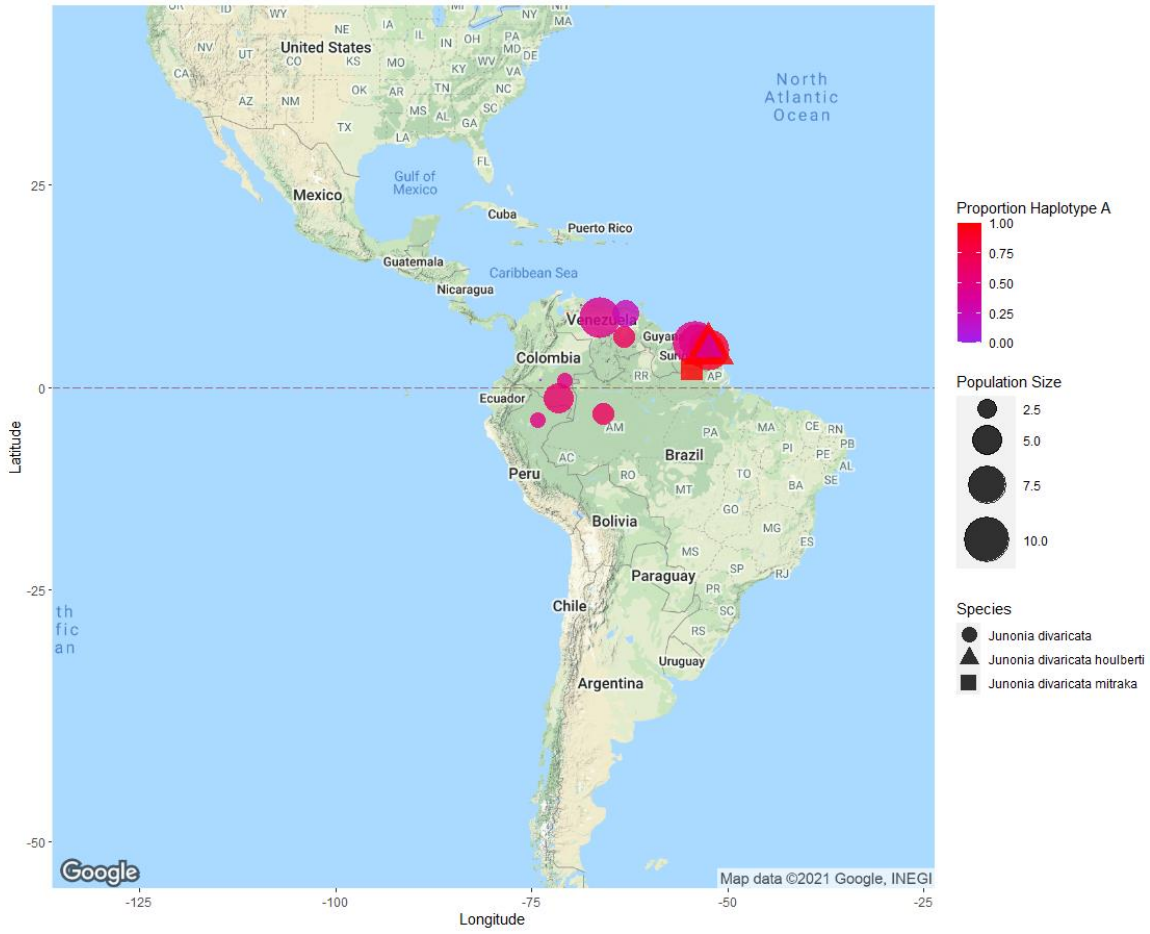

**Figure 2**. Whole geographic study area species distribution and proportion haplotype A for *Junonia evarete* (27 samples). Proportion of haplotype A is represented using a colour gradient with red being 100% group A and purple being 0% group A. Species designations are represented using different shapes and the population size is represented by the size of the shapes on the map.

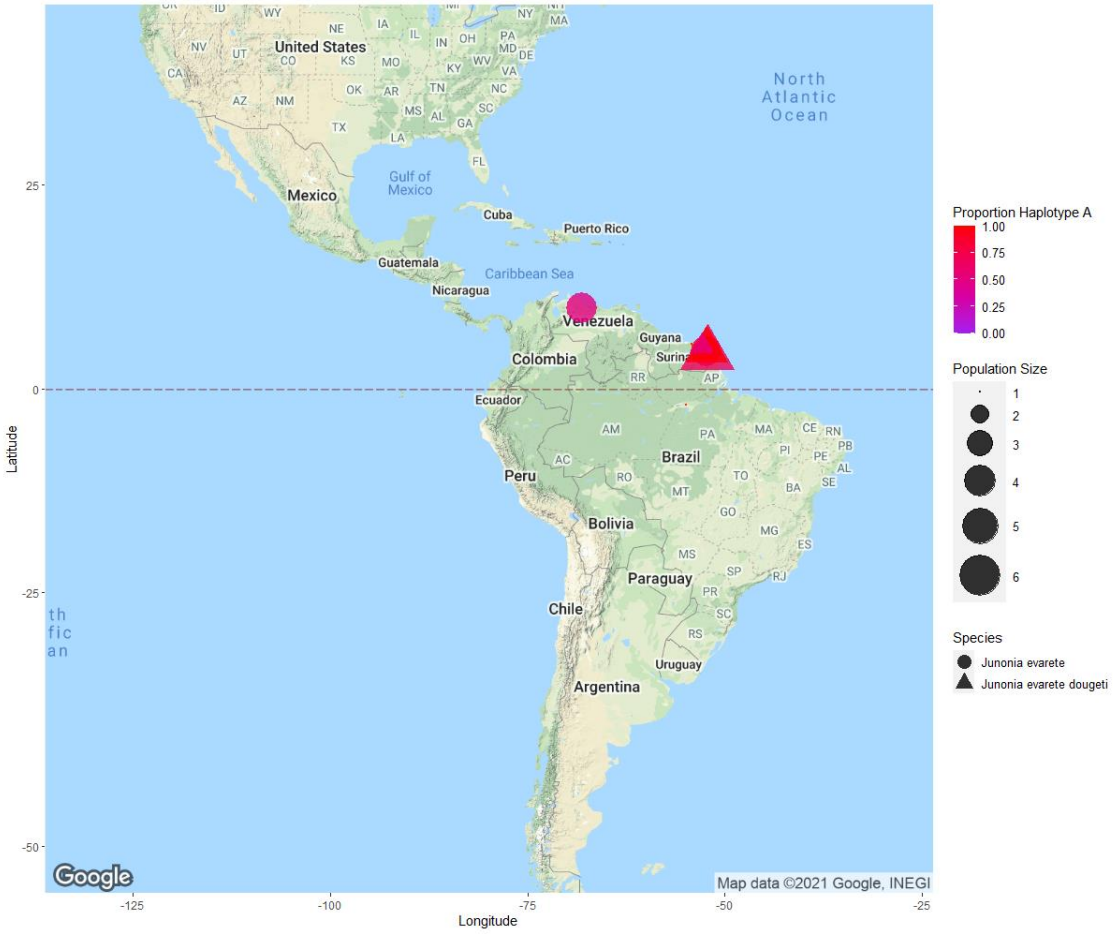

**Figure 3**. Whole geographic study area species distribution and proportion haplotype A for *Junonia flirtea* (25 samples). Proportion of haplotype A is represented using a colour gradient with red being 100% group A and purple being 0% group A. Species designations are represented using different shapes and the population size is represented by the size of the shapes on the map.

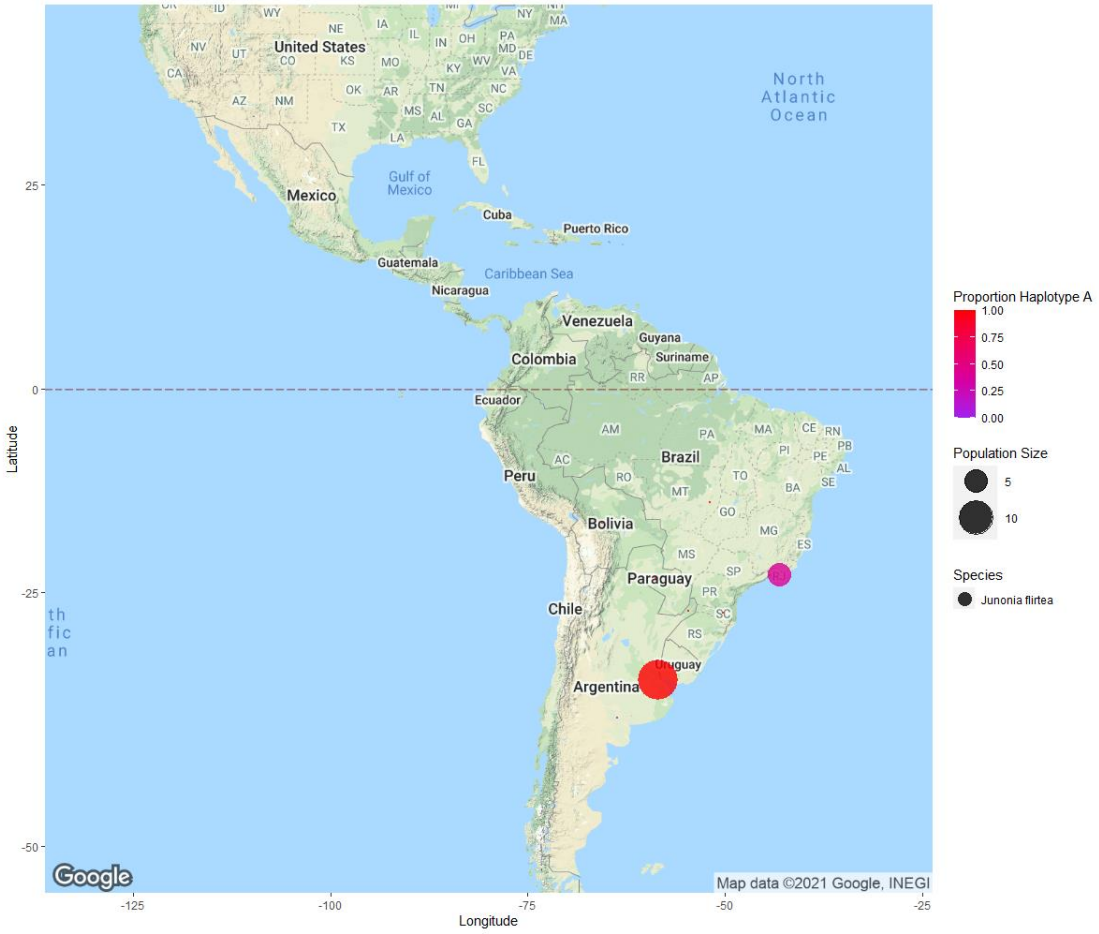

**Figure 4**. Whole geographic study area species distribution and proportion haplotype A for *Junonia fuscescens* (34 samples). Proportion of haplotype A is represented using a colour gradient with red being 100% group A and purple being 0% group A. Species designations are represented using different shapes and the population size is represented by the size of the shapes on the map.

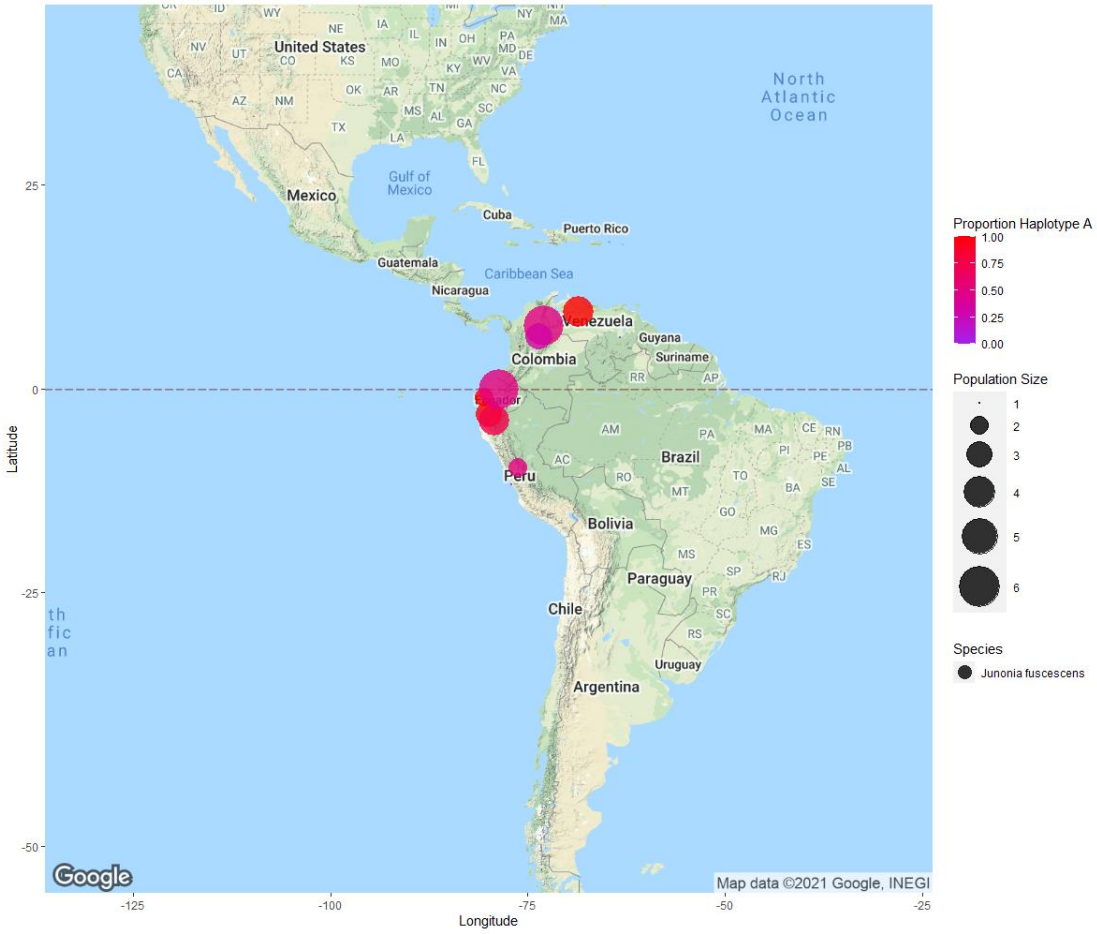

**Figure 5**. Whole geographic study area species distribution and proportion haplotype A for *Junonia genoveva* (317 samples). Proportion of haplotype A is represented using a colour gradient with red being 100% group A and purple being 0% group A. Species designations are represented using different shapes and the population size is represented by the size of the shapes on the map.

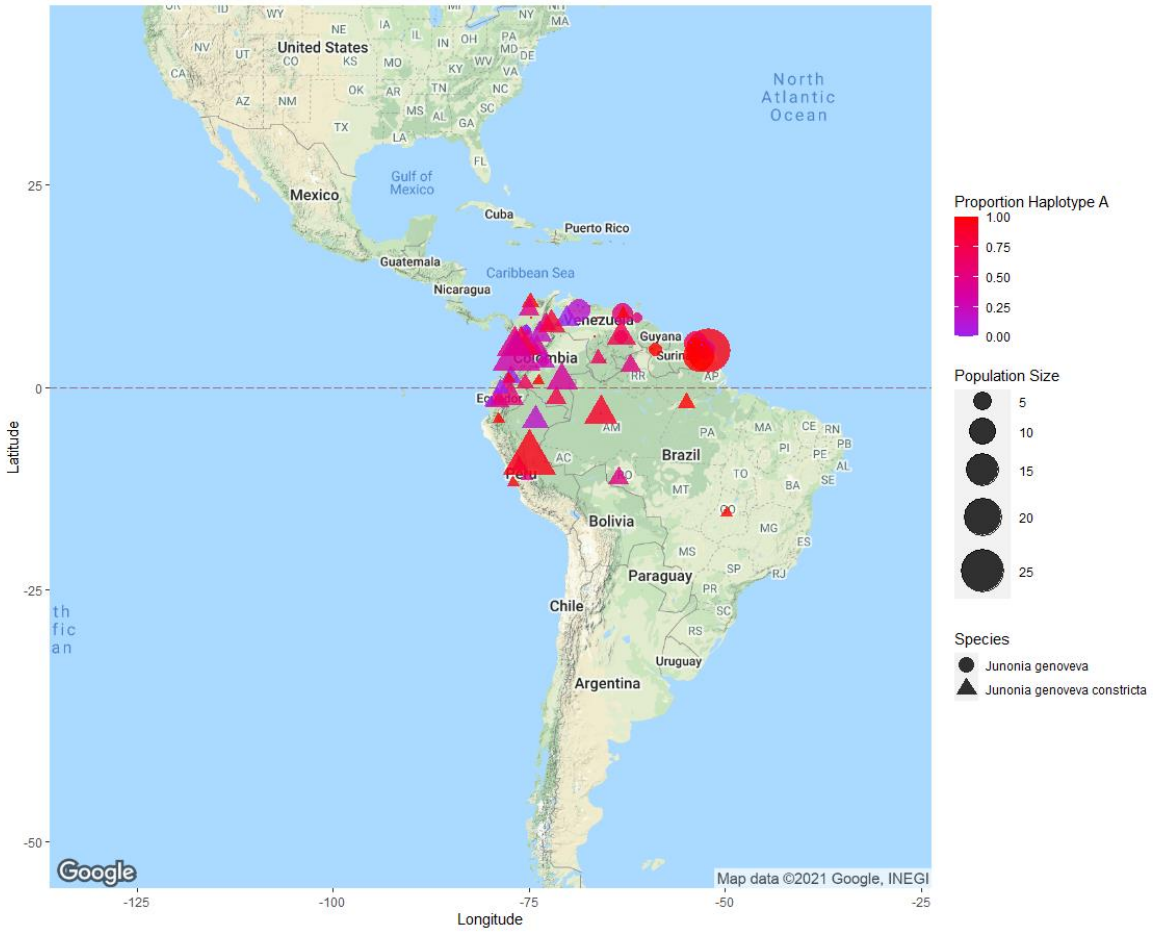

**Figure 6**. Whole geographic study area species distribution and proportion haplotype A for *Junonia grisea* (218 samples). Proportion of haplotype A is represented using a colour gradient with red being 100% group A and purple being 0% group A. Species designations are represented using different shapes and the population size is represented by the size of the shapes on the map.

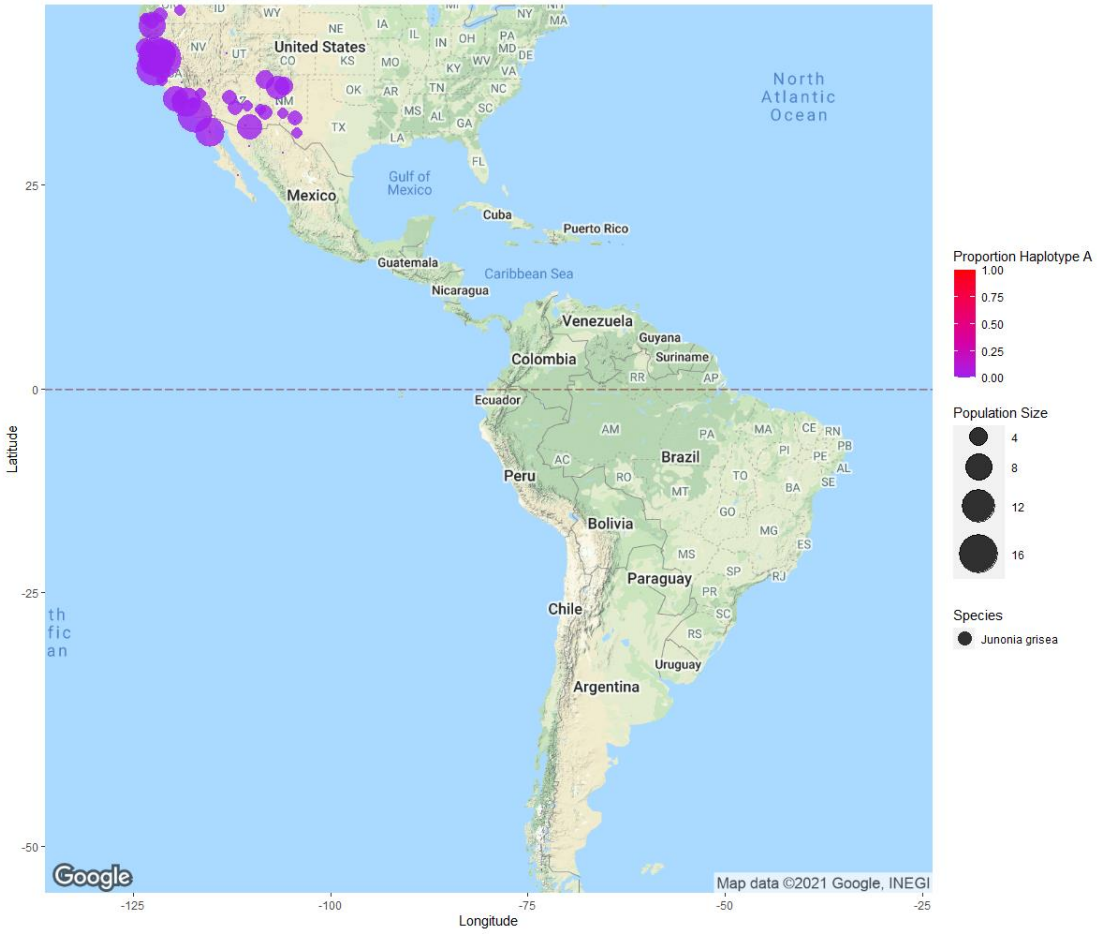

**Figure 7**. Whole geographic study area species distribution and proportion haplotype A for *Junonia hilaris* (31 samples). Proportion of haplotype A is represented using a colour gradient with red being 100% group A and purple being 0% group A. Species designations are represented using different shapes and the population size is represented by the size of the shapes on the map.

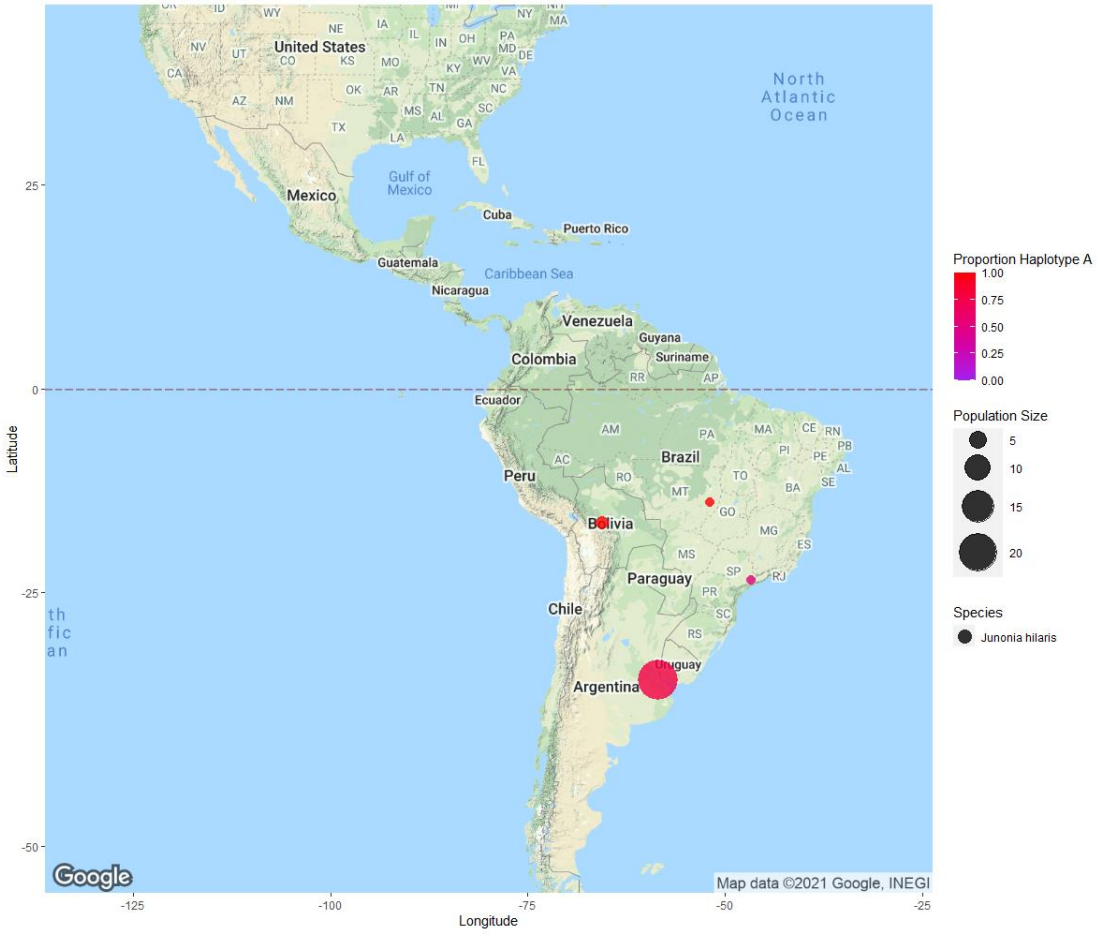

**Figure 8**. Whole geographic study area species distribution and proportion haplotype A for *Junonia infuscata* (17 samples). Proportion of haplotype A is represented using a colour gradient with red being 100% group A and purple being 0% group A. Species designations are represented using different shapes and the population size is represented by the size of the shapes on the map.

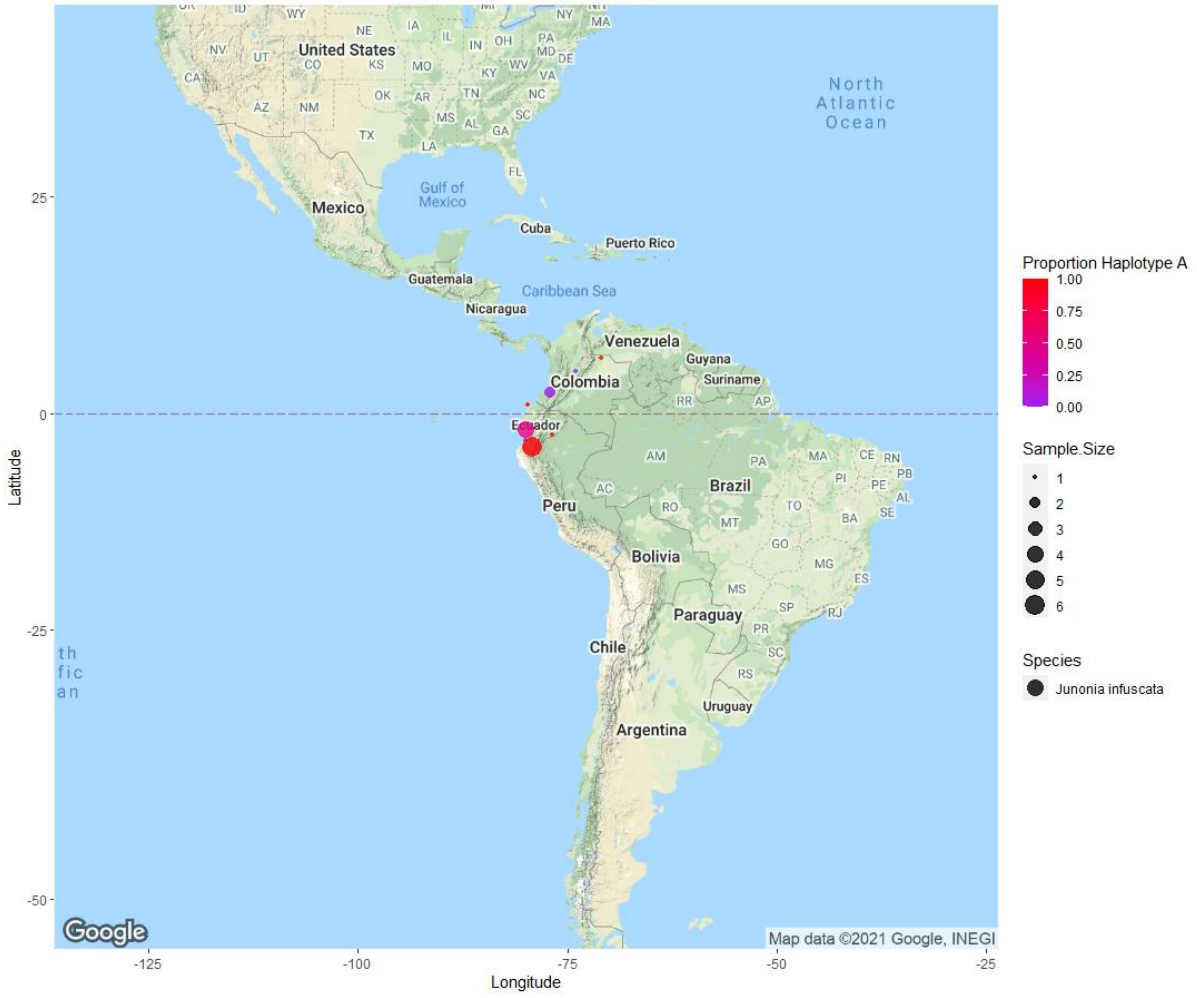

**Figure 9**. Whole geographic study area species distribution and proportion haplotype A for *Junonia litoralis* (27 samples). Proportion of haplotype A is represented using a colour gradient with red being 100% group A and purple being 0% group A. Species designations are represented using different shapes and the population size is represented by the size of the shapes on the map.

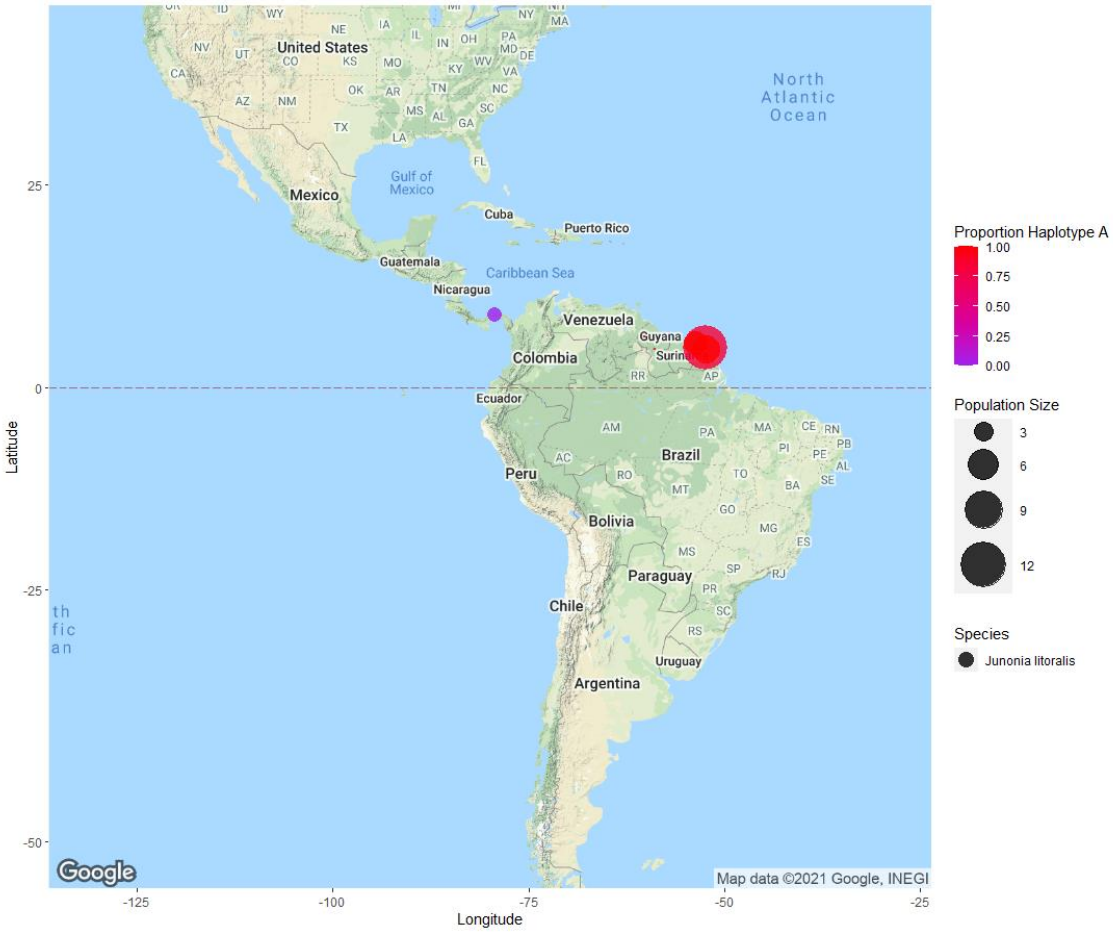

**Figure 10**. Whole geographic study area species distribution and proportion haplotype A for *Junonia neildi* (444 samples). Proportion of haplotype A is represented using a colour gradient with red being 100% group A and purple being 0% group A. Species designations are represented using different shapes and the population size is represented by the size of the shapes on the map.

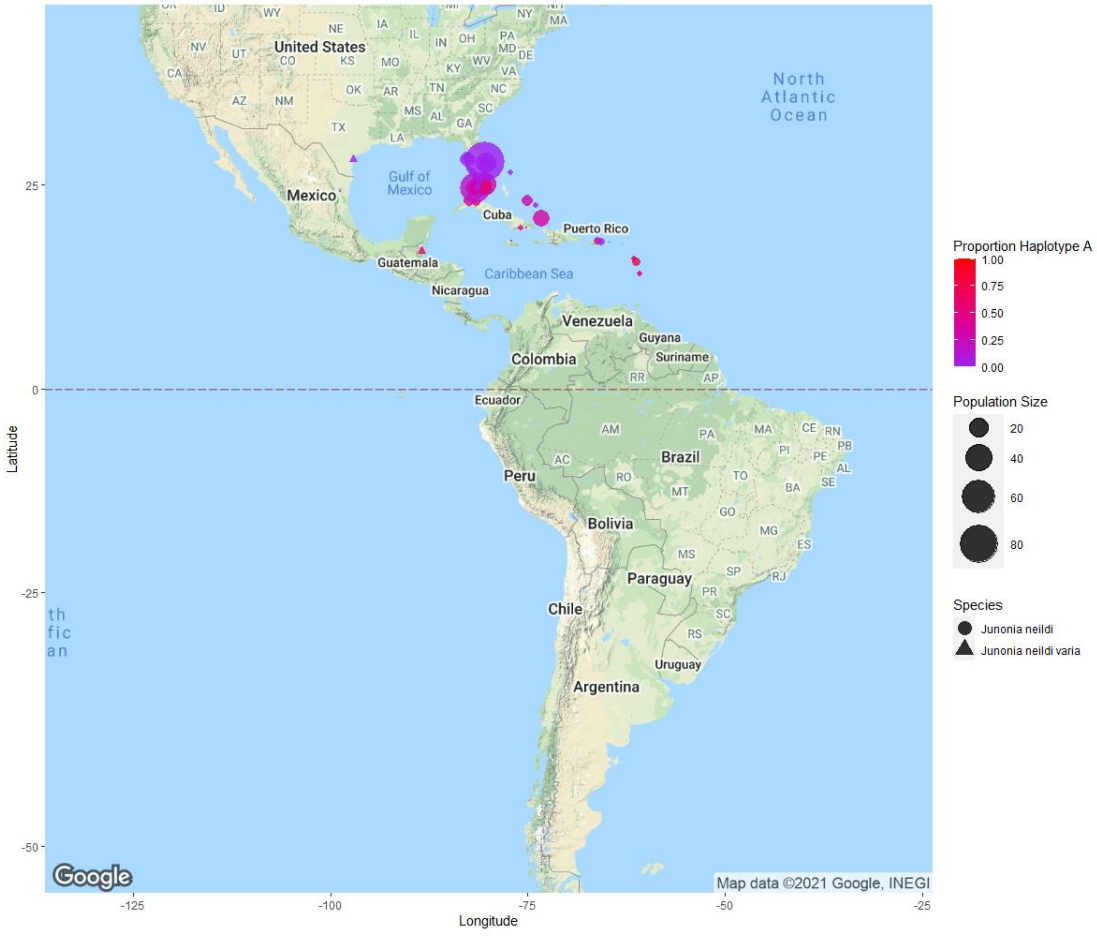

**Figure 11**. Whole geographic study area species distribution and proportion haplotype A for *Junonia nigralis* (7 samples). Proportion of haplotype A is represented using a colour gradient with red being 100% group A and purple being 0% group A. Species designations are represented using different shapes and the population size is represented by the size of the shapes on the map.

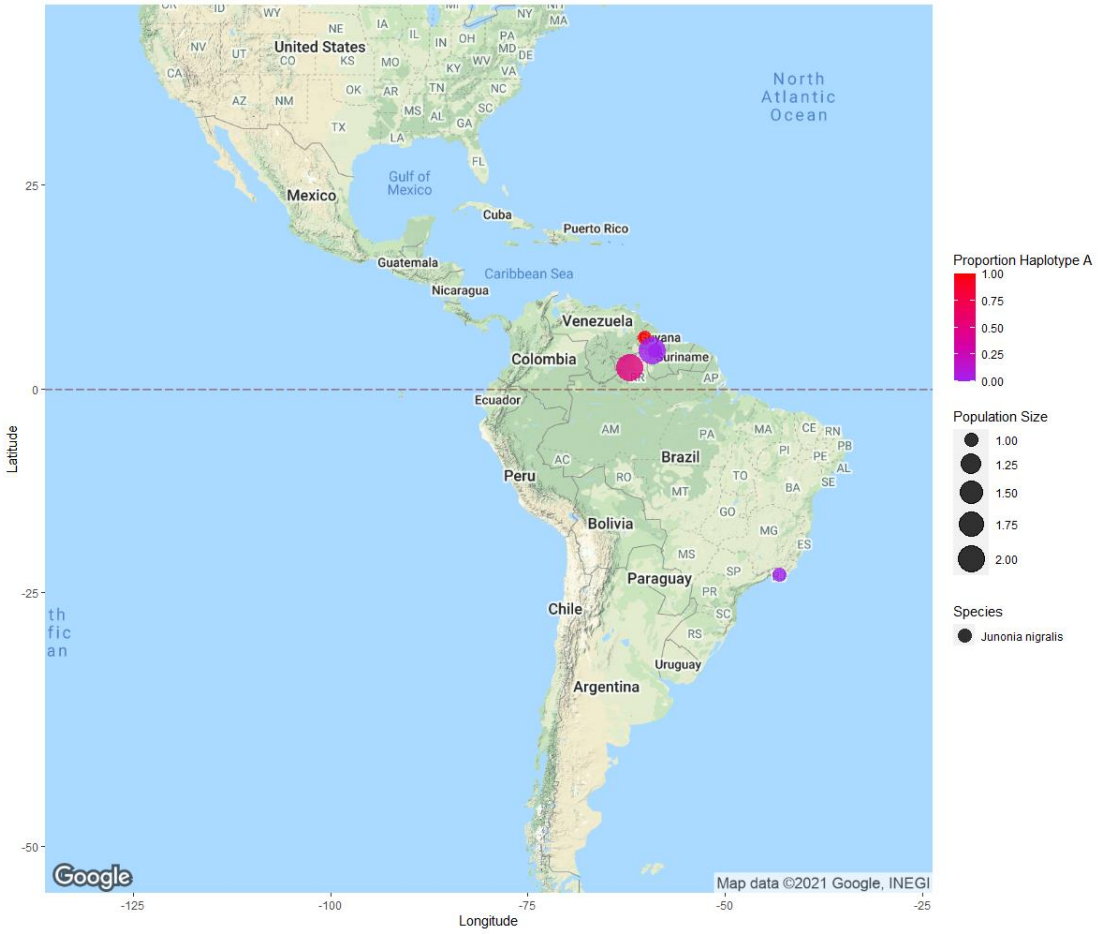

**Figure 12**. Whole geographic study area species distribution and proportion haplotype A for *Junonia nigrosuffusa* (157 samples). Proportion of haplotype A is represented using a colour gradient with red being 100% group A and purple being 0% group A. Species designations are represented using different shapes and the population size is represented by the size of the shapes on the map.

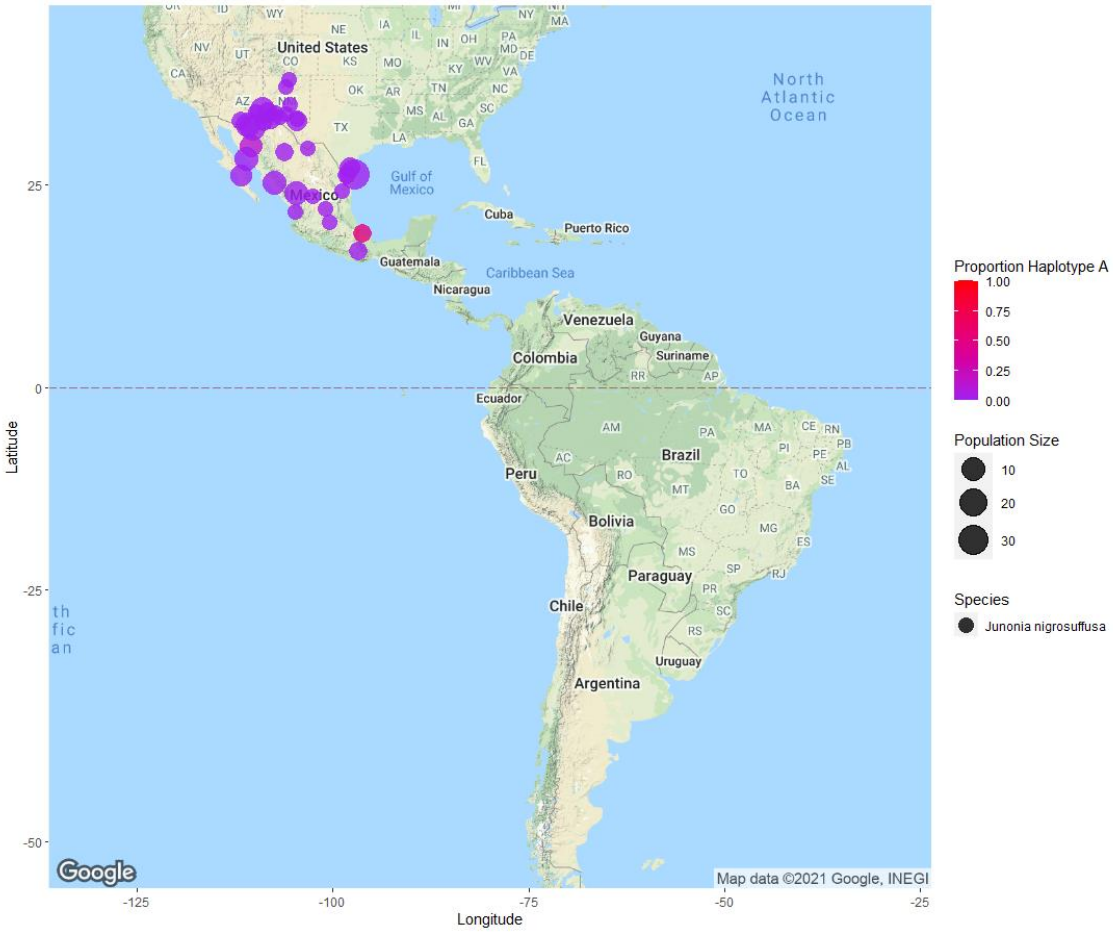

**Figure 13**. Whole geographic study area species distribution and proportion haplotype A for *Junonia oscura* (9 samples). Proportion of haplotype A is represented using a colour gradient with red being 100% group A and purple being 0% group A. Species designations are represented using different shapes and the population size is represented by the size of the shapes on the map.

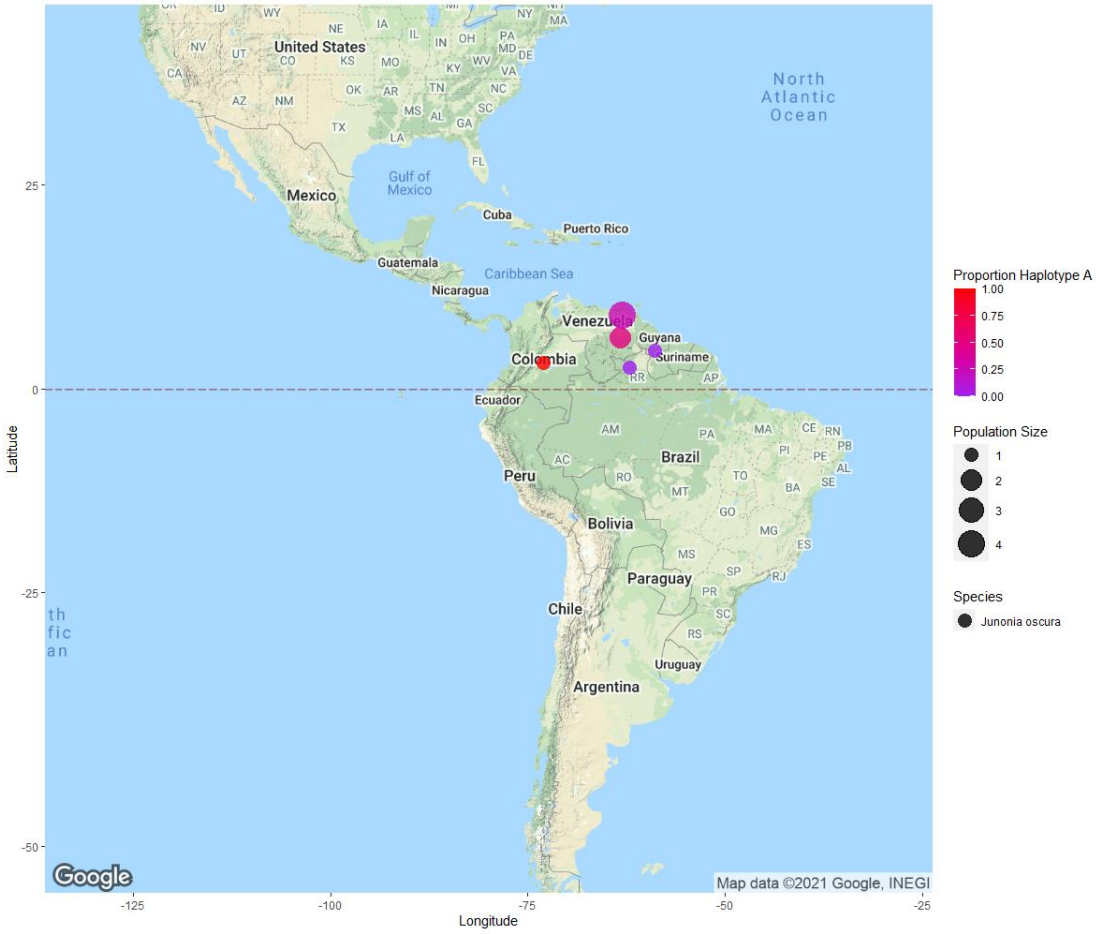

**Figure 14**. Whole geographic study area species distribution and proportion haplotype A for *Junonia pacoma* (28 samples). Proportion of haplotype A is represented using a colour gradient with red being 100% group A and purple being 0% group A. Species designations are represented using different shapes and the population size is represented by the size of the shapes on the map.

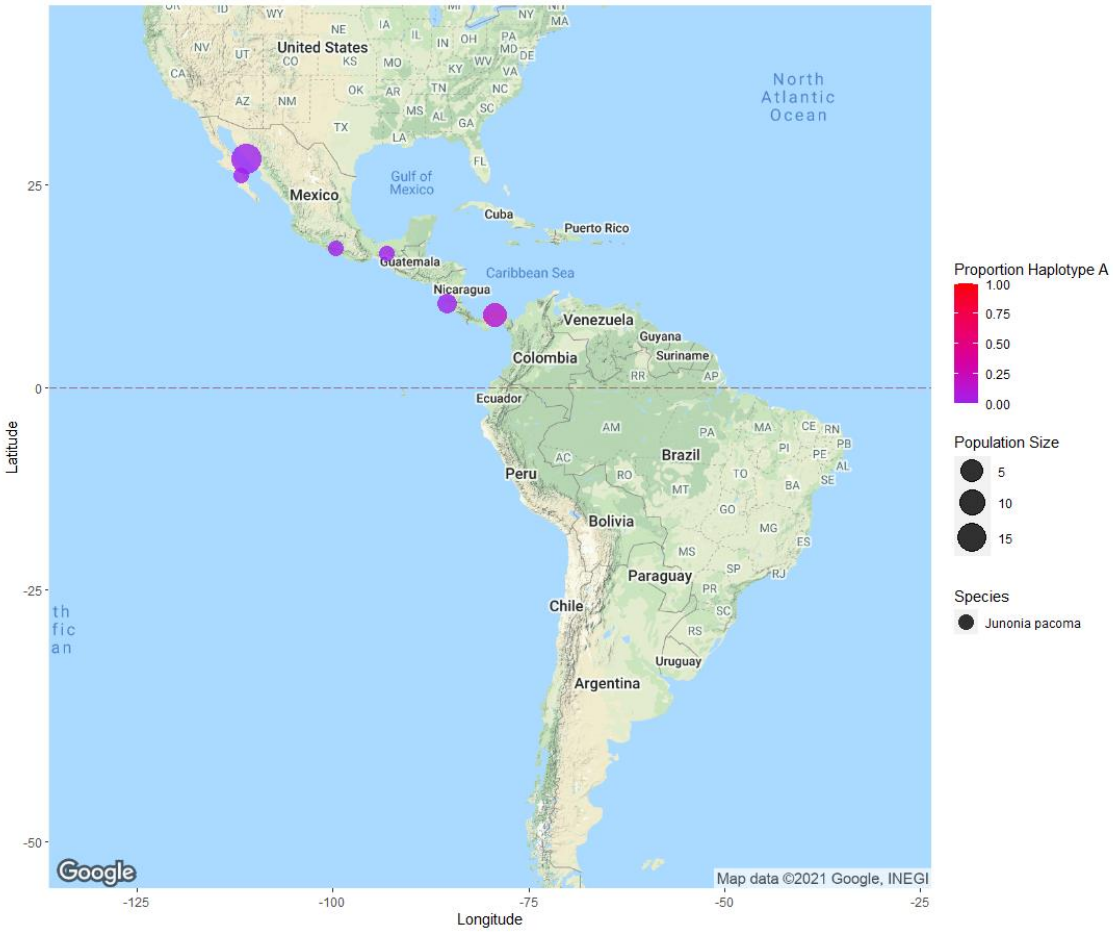

**Figure 15**. Whole geographic study area species distribution and proportion haplotype A for *Junonia wahlbergi* (116 samples). Proportion of haplotype A is represented using a colour gradient with red being 100% group A and purple being 0% group A. Species designations are represented using different shapes and the population size is represented by the size of the shapes on the map.

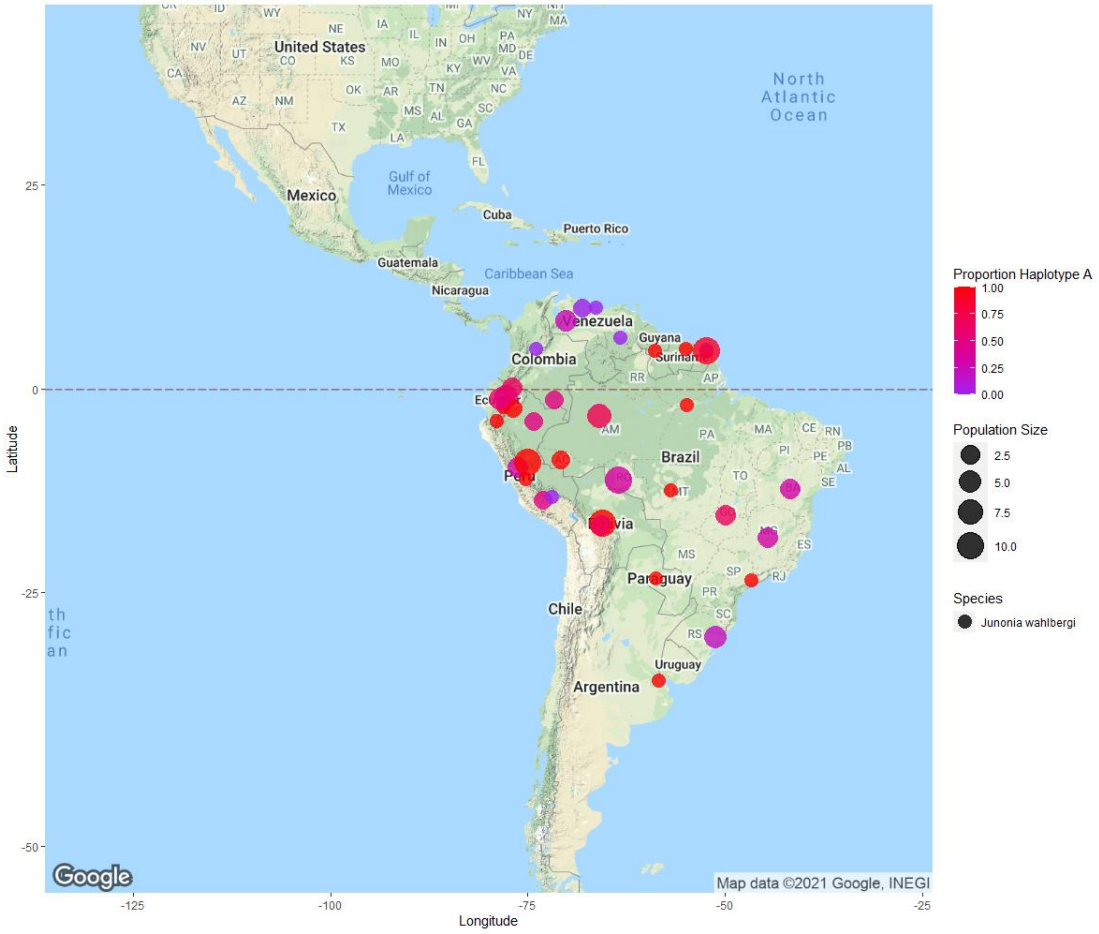

**Figure 16**. Whole geographic study area species distribution and proportion haplotype A for *Junonia zonalis* (498 samples). Proportion of haplotype A is represented using a colour gradient with red being 100% group A and purple being 0% group A. Species designations are represented using different shapes and the population size is represented by the size of the shapes on the map.