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Using conservation genetics to inform reintroduction of the endangered Mottled Duskywing (Erynnis martialis)

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Abstract

Habitat loss and climate change have caused declines in species diversity and abundance globally, including in butterflies which are important components of many ecosystems. Reintroductions are increasingly used to reverse diversity loss but are most effective when informed using genetics. I developed 24 microsatellites and characterized genetic structure and diversity of the endangered Mottled Duskywing (*Erynnis martialis*) in Ontario and neighbouring provinces and states. These were used to inform a planned reintroduction in Ontario. Populations had moderate levels of genetic diversity, however all but the largest populations may be subject to appreciable levels of genetic drift. Populations more than 8 km apart appear to be isolated from each other. My work forms part of a larger effort to achieve the overall recovery of the species in Ontario. Tools I developed may be used to inform future reintroductions of the species, and to monitor status of introduced and extant populations.

Keywords

Species at-risk, reintroduction, Mottled Duskywing, conservation genetics, microsatellites, molecular ecology

Summary for Lay Audience

A growing human population and associated climate changes and habitat losses have led to declines in species across the globe, with some researchers calling this the sixth mass extinction event. Worldwide declines in insect species threaten a “catastrophic collapse of nature’s ecosystems”. Butterfly species are facing one of the largest of these declines. Butterflies are important as they provide ecological services and are important indicators of environmental health. Re-introducing species to areas where they once occurred is increasingly being used as a strategy for recovering at-risk populations. Many early reintroductions of butterfly species were largely unsuccessful due to a lack of background research and rigorous protocols. The Mottled Duskywing (*Erynnis martialis*) is a medium-sized, brown butterfly listed as Endangered in Ontario, and reintroductions to formerly occupied locations have begun as part of the species Recovery Plan. I developed and used genetic tools to determine metrics of diversity within and among Mottled Duskywing populations in Ontario and nearby locations. Genetic diversity is an indicator of population health; the more variability there is among individuals of a population, the more likely that a population will be able to survive in a changing environment. Populations had moderate amounts of diversity and differed from one another. However, populations within 8 km of one another were not differentiated. These results have informed a reintroduction of the species to Pinery Provincial Park through the selection of the source population based on the levels of genetic diversity in the populations. Moving forward in the recovery goals, the tools I developed can be used to inform future reintroductions through the selection of other potential source populations

and selection of females used in captive rearing programs. Additionally, they can be used to assess the genetic status of both remnant and reintroduced populations.

Co-Authorship Statement

Chapters 2 and 3 of this thesis are planned manuscripts to be submitted to peer-reviewed journals. Chapter 2 will be submitted as a primer note.

Both manuscripts resulting from my thesis chapters will be co-authored with my supervisor Nusha Keyghobadi, and collaborative project leads Jessica Linton, Ryan Norris, Gard Otis, and Adrienne Brewster. Chapter 3 will also include Richard Westwood as a co-author.

For all chapters, I contributed to all components of project completion including research conception and design, sample collection, data collection, lab work, genotyping, data analysis and interpretation, and all writing. For all chapters, NK contributed to research conception and design, supervision through all components of the research, and substantial feedback on writing components, as well as research funding. For all chapters, JL, RN, GO, and AB also contributed to study conception and research funding. For chapter 2, sampling was overseen by me, NK, GO, and JL. Full butterfly bodies for microsatellite development were provided by the captive rearing program at the Cambridge Butterfly Conservatory (AB). For chapter 3, sampling was overseen by me, NK, GO, JL, AB, and RW.

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List of Symbols and Abbreviations

List of Symbols

bp .... base pairs

°C .... degrees Celsius

cm^2 .... centimeters squared

km.. kilometers

mg .... milligrams

min .... minutes

mL.. milliliters

mM .... millimolar

s .... seconds

U .... enzyme unit

µL .... microliter

µM.. micromolar

X.. times

% .... percent

List of Abbreviations

A_R .... allelic richness

BSA .... bovine serum albumin

DAPC .... Discriminant Analyses of Principal Components

DNA .... deoxyribonucleic acid

dNTPs .... deoxyribonucleotide triphosphates

F_{ST} .... fixation index

GEA..Genome Environment Analyses

H_E …….……..……. expected heterozygosity

H_O …….……..……. observed heterozygosity

HWE ……. Hardy Weinberg Equilibrium

IBD …….....… Isolation by Distance

IBE …….....… Isolation by Environment

IUCN ..... International Union for the Conservation of Nature

MgCl ……..... magnesium chloride

N_e …….....……..... effective population size

PCR ……....……. polymerase chain reaction

SNP…….. single nucleotide polymorphism

List of Software and Software Packages

List of Software

ArcGIS Pro: https://www.esri.com/en-us/arcgis/products/arcgis-pro/overview

BOTTLENECK: http://www1.montpellier.inra.fr/CBGP/software/Bottleneck/bottleneck.html

GeneMarker ® HID: https://softgenetics.com/GeneMarkerHID.php

GENEPOP: https://genepop.curtin.edu.au/

Google Earth Pro: https://support.google.com/earth/answer/21955?hl=en

Micro-Checker 2.2: https://micro-checker.software.informer.com/2.2/

NeEstimator: http://www.molecularfisherieslaboratory.com.au/neestimator-software/

PrinSeq: http://prinseq.sourceforge.net/

R version 4.1.3: https://www.r-project.org/

RStudio: https://www.rstudio.com/

STRUCTURE 2.3.4: https://web.stanford.edu/group/pritchardlab/structure_software/release_versions/v2.3.4/html/structure.html

List of R Packages

adegenet: Jombart 2014

diveRsity: Keenan 2017

ecodist: Goslee and Urban 2007

hierfstat: Goudet 2005

pegas: Paradis 2010

Chapter 1

1 General Introduction

1.1 Biodiversity Loss

The Earth is currently experiencing a rapid and widescale loss of biodiversity that poses a multi-faceted threat to humans; biodiversity provides valuable resources such as medicines and new food sources, access to clean water and air, and ecosystem stability in the face of environmental change (Díaz et al. 2006). Prominent scientists have argued that there is no possibility of humans' survival on Earth without the maintenance of a substantial fraction of the existing biodiversity (Hanski 2005). Yet, biodiversity loss is occurring at alarming rates, recent targets set to reduce biodiversity loss have not been met, and these trends are predicted to continue (Waldron et al. 2017). This has led many researchers to conclude that the Earth is entering a sixth mass extinction, with current extinction rates estimated to be at least a hundred times higher than the background rates (Ceballos et al. 2015). This elevation in extinction rate is due, both directly and indirectly, to human activities. It is now increasingly accepted that we have entered a new era called the "Anthropocene", as humans have become the dominant evolutionary force on the planet (Pievani 2014).

Fragmentation, degradation, and loss of suitable habitat due to growing human populations and climate change are cited as two of the major factors that have caused declines in global biodiversity (Oliver and Morecroft 2014). When their habitats are lost, populations of dependent species are also likely to decline (Brown et al. 2018) or become extinct (Jetz et al. 2007). Habitat fragmentation can create small, isolated populations

increasing the risk of extinction (Burkey and Reed 2006). Habitat quality, size, and connectivity are all important for the maintenance of species (Isaak et al. 2007).

Additionally, the Intergovernmental Panel on Climate Change (IPCC) predicts significant changes to global temperature and precipitation patterns by the year 2100 (IPCC 2014). Research has shown that climate change has various effects on species' ecology, such as range shifts and reductions, and phenological shifts such as changes to butterfly emergence times (Parmesan 2006). Climate change contributes to extinction when species cannot adapt or migrate rapidly enough to keep pace with the shifting environmental conditions (Feeley et al. 2012). A modelling study of 540 species in 17 clades of terrestrial vertebrates found that temperatures are rising due to anthropogenic climate change faster than most species can adapt (Quintero and Wiens 2013).

Interactions between habitat loss and climate change may exacerbate the impacts of either of these forces in isolation (Mantya-Pringle et al. 2012). For example, populations that have become smaller due to habitat fragmentation may be more susceptible to environmental changes caused by climate change (i.e., the small population paradigm; Brook et al. 2008). Furthermore, habitat fragmentation may limit the ability of species to migrate and shift their range boundaries in response to climate change (Dyer 1995). Most studies indicate that habitat loss is a larger threat to global biodiversity (Mantya-Pringle et al. 2012). However, the impact of climate change is predicted to worsen (Lemoine et al. 2007; Pimm 2008). Additionally, modelling predicts that future climate change will impact species that are largely unaffected by human activities to date (Pimm 2008).

1.1.1 Butterfly species declines

Insects are the most diverse group of organisms on the planet; they represent more than half of the known terrestrial species in the animal kingdom (Stork 2018). Moreover, it is estimated that there are millions of species of insects that have yet to be described (Wilson 1987). Humans rely on insects for essential services such as decomposition, pollination, pest control, and more (Losey and Vaughan 2006). Worldwide declines in insect species threaten a "catastrophic collapse of nature's ecosystems" (Sánchez-Bayo and Wyckhuys 2019). Despite the vital role that invertebrates play in the function of ecosystems, these declines have received significantly less attention than those of mammals and other charismatic megafauna (Prather et al. 2013). Additionally, past research has shown that of thousands of insect extinctions predicted to have occurred, only 70 of them were documented (Dunn 2005). Many insect extinctions may go unnoticed due to a lack of monitoring, scientific description, and identification of invertebrate species (Eisenhauer et al. 2019).

Butterflies are experiencing some of the largest known declines among the insects, with more than half of Lepidoptera species that have been surveyed declining worldwide (Sánchez-Bayo and Wyckhuys 2019). A study by Dirzo et al. (2014) reported strong evidence of a 35% decline in abundance of Lepidoptera species over 40 years. The leading causes of butterfly declines are habitat loss/degradation, chemical pollution, and climate change (Warren et al. 2021). Previous studies have found that the ranges of butterfly species have been retracting as climate has deteriorated, in some cases leading to population-level extinctions (Thomas et al. 2006). Other threats include exotic plant and animal invasion (Wagner and Van Driesche 2010), natural system modifications

(Geyle et al. 2021), and genetic factors associated with small populations (Schmitt and Hewitt 2004). Conservation strategies that have been implemented aimed at protecting butterflies include restoration of habitat, captive rearing, and reintroductions (Schultz et al. 2008).

Butterflies are important components of ecosystems and often thought of as bio-indicators due to their visibility and sensitivity to changes in the environment (Pe'er and Settele 2008). Many consider butterflies the 'poster child' of the invertebrate world (Schultz et al. 2008). They are commonly used as a model species in ecological studies and have been used as indicators of habitat quality (Uehara-Prado and Freitas 2009), species richness (Fleishman et al. 2005), and climate change impact (Vickery 2008). Additionally, they have provided foundational information on metapopulation dynamics which have had important implications for conservation (Hanski et al. 1995; Ehrlich 1992). Butterflies have been found useful as an 'umbrella taxon', where protection of butterflies may ensure the protection of other threatened organisms in the same ecosystems (Betrus et al. 2005). In this thesis, I undertake a population genetic study of the only endangered butterfly in the province of Ontario, the Mottled Duskywing, *Erynnis martialis* (Scudder 1870), to information conservation and restoration efforts for this species.

1.1.2 Genetic diversity as a component of biodiversity

The International Union for the Conservation of Nature (IUCN) recognizes three levels of biodiversity: ecosystems, species, and genetics (McNeely et al. 1990). Often, conservation work focuses on the ecosystem and species levels because they are more

visible. Additionally, ecosystem services such as crop pollination make the conservation of species and ecosystems attractive to funding agencies (Spangenberg and Settele 2010). However, genetic diversity, defined as heritable variation among individuals and populations within a species (Rao and Hodgkin 2002), is also of critical importance. Genetic diversity allows populations and species to adapt to environmental change (Lande and Shannon 1996), such as emerging diseases (O'Brien and Evermann 1988), climate change (Ehlers et al. 2008), and other disturbances (Hughes and Stachowicz 2004). Furthermore, a lack of genetic diversity can lead to dire problems in populations such as inbreeding depression (Charlesworth and Charlesworth 1999), which I discuss in detail later. Importantly, inbreeding depression can lead to increased risk of extinction, making it extremely important for the conservation of species (Saccheri et al. 1996; Charlesworth and Charlesworth 1987).

1.2 Population genetic patterns and processes

Population genetics is defined as the study of genetic variation within a locus or loci, among individuals of a population or populations, and provides the conceptual and analytical framework for understanding the causes and consequences of loss of genetic diversity (Hedrick 2011). Genetic variation is influenced by four main genetic processes: natural selection, mutation, gene flow, and genetic drift (Star and Spencer 2013; Wright 1931). Natural selection is a non-random difference among individuals in fitness, due to advantages (or disadvantages) of heritable and variable traits in a particular environment and leads ultimately to an increase in the frequency of beneficial alleles (or decrease in detrimental alleles) from one generation to the next (Gregory 2009). When similar

selection pressures are acting on different populations, those populations will tend to become more similar in allele frequencies (Hendry et al. 2002). Similarly, populations experiencing different selection pressures are likely to diverge in allele frequencies. A mutation is a change in DNA sequence that can result from DNA replication errors, exposure to radiation or mutagens, or infection by viruses (Brown 2002). Selection acts on the variation that is introduced by mutations, such that in any given context mutations can be advantageous and increase fitness, deleterious and decrease fitness, or neutral (Loewe and Hill 2010). Gene flow is the movement of genes from one population to another (McDermott and McDonald 1993). Gene flow between two populations can introduce new genetic variation into a population and causes those populations to become more similar in allele frequencies (Slatkin 1985). Genetic drift is a process by which the frequencies of alleles in a population change by chance over time, due to the random sampling of alleles from a parental population to produce the offspring generation (Charlesworth 2009). Genetic drift is more pronounced the smaller a population is, and it both reduces the amount of genetic variation within populations and, on average, increases divergence among populations (Andrews et al. 2017).

1.2.1 Population structure and genetic differentiation

Population genetic structure is the amount and distribution of genetic variation within and among different populations (McDermott and McDonald 1993). While genetic diversity within populations is critical for both evolution and conservation, genetic variation or differentiation among different populations is also key because it determines whether populations are linked or may be on separate evolutionary trajectories (Duminil et al. 2007). Genetic structure and differentiation among spatially separated populations

can be shaped by geographic (e.g., isolation by distance; IBD), environmental (e.g., isolation by environment; IBE), and historic factors (e.g., historical distribution of populations and habitats) (Xu et al. 2017). Both ecological and geographical factors can reduce gene flow between populations leading to divergence (Wang et al. 2013).

Isolation by distance is a positive correlation between genetic and geographic distances of populations, and results when dispersal and gene flow become increasingly limited as the spatial separation of populations increases. Dispersal is the movement of individuals from one population to another (Broquet and Petit 2009) and leads to gene flow if an individual or its offspring breeds with a member of the population to which it has moved (Deere et al. 2017). When populations are isolated by habitat fragmentation and degradation, and individuals are unable to disperse between populations, the populations experience decreased gene flow (Miles et al. 2019). Decreased gene flow, acting in combination with genetic drift, is expected to reduce genetic diversity within the populations and increase genetic differentiation among them (Munshi-South et al. 2016). When gene flow among populations becomes so reduced that it can no longer counter the effects of genetic drift, patterns of IBD also break down (Hutchison and Templeton 1999).

Isolation by environment is a correlation between genetic distance and environmental dissimilarity (Sexton et al. 2014). Isolation by environment can occur when populations occupy different environments that generate divergent selection pressures, which reduces gene flow between those populations (Wang and Bradburd 2014). Isolation by distance and environment are particularly important to understand for

at-risk species as they can inform conservation strategies, specifically those directed at managing genetic diversity (Xu et al. 2017).

1.2.2 Demographic stability and genetic variation

Broadly defined, demography is the study of the characteristics of populations, and of factors that influence population growth or decline (Tarsi and Tuff 2012). Demographic parameters relevant to population genetics include population size, survival rates, age structure, sex ratio, density, population growth rates, historical population size changes, and exchange of individuals among populations (Tarsi and Tuff 2012; Shen et al. 2019; Lowe et al. 2017; Hughes et al. 2017). These demographic parameters can affect variation in individual fitness, as well as drift and gene flow, in turn determining patterns of genetic variation. Conversely, observed patterns of genetic variation within and among populations can be used to make indirect inferences about these demographic parameters, and the processes they represent, thereby providing insight into the risk of extinction faced by a given population (Tarsi and Tuff 2012).

1.2.2.1 *Effective population size*

The most fundamental demographic parameter is the number of individuals in a population, also known as the census population size (N; Lebreton et al. 1992). The effective population size (N_e) is a related and central concept in population genetics and is defined as the number of individuals in an idealized population (i.e., each individual makes an equal reproductive contribution to the next generation) that experiences the same level of genetic drift (i.e., the same effect of random sampling of alleles) as that in the actual population (Kimura and Crow 1963; Plutysnki 2007). Effective population

sizes are often much smaller than census population sizes (Husemann et al. 2016) due to differential reproductive success among individuals, unequal sex ratio, and fluctuating population size (Palstra and Ruzzante 2008). Effective population size reflects how strongly drift acts in a population, and therefore how vulnerable the population is to loss of genetic diversity over time.

1.2.2.2 *Demographic bottlenecks*

A demographic or population bottleneck is an event that drastically, but temporarily, reduces the census size of a population. Demographic bottlenecks can cause a decrease in the gene pool due to loss of alleles (Wright 1951). This can occur even if a population experiences only a brief period of reduced size (Maruyama and Fuerst 1985), although the loss of allelic diversity is greater the longer and more drastically the census population size is decreased. Detecting recent population bottlenecks is relevant for at-risk species as bottlenecks not only increase the risk of population extinction due to decreased genetic diversity (Luikart et al. 1998) but may also reflect increased vulnerability to demographic stochasticity.

1.2.3 Microsatellites

The genetic variation within and among populations can be quantified and described with the use of molecular or genetic markers. One of such markers, microsatellites, are tandem repeats of short (1-6 base pairs) DNA sequence motifs that are located randomly throughout the genome and thought not to perform any function (i.e., are non-coding DNA) (Vieira et al. 2016, Ellegren 2004). Different alleles at a microsatellite locus have a different number of repeats of the motif. Microsatellites have high mutation rates that

often arise through replication slippage (Bhargava and Fuentes 2010) and are therefore highly variable. Because they are non-coding, microsatellites are also likely to be selectively neutral. Additionally, they are bi-parentally inherited and co-dominant meaning that both maternal and paternal DNA are represented, and all homozygote genotypes can be distinguished from heterozygote genotypes (Sah et al. 2021). These characteristics make microsatellites useful for determining genetic diversity and population structure, as well as assessing identity, population of origin, paternity, and kinship of individuals (Chistiakov et al. 2006).

Microsatellites have been used extensively to inform conservation programs (Hedrick 2001), including in Lepidoptera such as the endangered Karner Blue Butterfly, for which microsatellites were used to analyze heterozygote deficiencies, geographic variation, and population structure (Anthony et al. 2001). They are particularly useful for studies of endangered species, as they can be analyzed from very small amounts of tissue or other biological material that can be collected with minimal sampling effort and disturbance to the species (Hansen et al. 2001).

Only 12 whole butterfly samples from a single population that had never been described genetically were available for marker development for this project (see Chapter 2). Therefore, microsatellites were a practical option considering their high variability. While I also considered using single nucleotide polymorphisms (SNPs), the potential for low diversity among the samples available for marker development meant a risk of not locating enough SNP loci to conduct robust analyses, as well as potential biases when applying those loci to other populations.

1.3 Species reintroductions

Unprecedented loss of species has led to an imperative to conserve the biodiversity that remains. Reintroduction is one approach to conserving biodiversity and is defined by the IUCN as “the intentional movement of an organism into a part of its native range from which it has disappeared or become extirpated in historic times” (IUCN 1987). Human-mediated species reintroduction or translocation is increasingly used as a strategy for population recovery, including for at-risk lepidopteran species (Daniels et al. 2018). Although much less effort has gone into invertebrate reintroductions compared to mammals (Jule et al. 2008, Jourdan et al. 2019), examples of the former are becoming more common (Schultz et al. 2008; Andersen et al. 2014; Dumeier et al. 2020; Kelemen and Rehan 2021). The goal of reintroduction is to establish a new population that is self-sustaining, has a low probability of extinction (Fraser 2008), and requires minimal long-term management (IUCN 1998). However, there is still no general agreement on the criteria for a successful reintroduction (Gusset 2009). Some suggested metrics of success are survival of the release generation, breeding by the release generation, and persistence of the re-established population (Seddon 1999).

Early reintroductions were often unsuccessful due to a lack of monitoring and documentation, and a lack of consideration or management of key factors such as habitat restoration and predators (Short et al. 1992). The IUCN created a set of guidelines for reintroductions to reduce the number of failed reintroductions (IUCN/SSC 2013). The guidelines recommend considering feasibility assessments and collection of sufficient background knowledge on the species and emphasize comprehensive monitoring and

documentation at all stages. Genetic monitoring has also become a useful tool in determining efficacy of reintroduction programs; using population genetics, changes in demographic parameters such as genetic structure and differentiation can be investigated and quantified (De Barba et al. 2010). The IUCN guidelines also point to an important role for genetic information and considerations that include adequate genetic diversity in both founders and the population(s) from which they are sourced, balancing the risks of inbreeding versus outbreeding among founders, and genetic provenance or match to the new location or environment (IUCN/SSC 2013).

1.3.1 Strategies for source population selection

1.3.1.1 *Genetic considerations for reintroduction: inbreeding depression*

If founders for a new population are chosen from a source population that is small, or if too few individuals are introduced, the new population will have a small effective size and low genetic diversity (Tracy et al. 2011). This in turn will result in inbreeding, defined as mating of individuals that are genetically similar to each other (Charlesworth and Charlesworth 1999). Inbreeding increases homozygosity, which can lead to an increased likelihood of deleterious recessive alleles persisting in homozygous form (Lynch 1991) and a decrease in variants that are maintained by heterozygote advantage (Hedrick and Garcia-Dorado 2016). These factors, in turn, can reduce fitness parameters such as survival and longevity (Hansson et al. 2001). This reduction in fitness is termed inbreeding depression and has been shown to increase likelihood of population extinction (Saccheri et al. 1996, Charlesworth and Charlesworth 1987).

Inbreeding depression is a concern in the management of endangered species as these species frequently have small, fragmented populations (Hedrick & Kalinowski 2000). Isolated populations that are experiencing little gene flow may have decreased fitness from the negative impacts of increased levels of genetic drift (Miles et al. 2019). While it is also possible for small, inbred populations to purge deleterious recessive alleles, resulting in a population that is well adapted to their current environment, this may result in the population being less capable of adapting to changes in the environment (van der Valk et al. 2021). Additionally, genetic purging occurs over long evolutionary time frames and is largely observed in species with historically small population size and low genetic diversity. Inbreeding depression is of particular concern in reintroduced populations because of the founder effect, which is "the establishment of a new population by a few original founders... which carry only a fraction of the genotypes of the parental population" (Mayr 1963). This effect promotes inbreeding and can enhance the rate of divergence from other populations in the species, which is more pronounced in short-lived species such as insects (Templeton 1980).

Research has shown that inbreeding can have different effects depending on the organism. For example, some invertebrates can thrive at levels of inbreeding that would result in inbreeding depression in mammals (Haikola et al. 2001). However, inbreeding has contributed to the extinction of wild butterfly populations (Frankham and Ralls 1998). Inbreeding has also been shown to have negative impacts on fitness of a butterfly species bred in captivity, with effects such as large decreases in egg hatching (Saccheri et al. 1996) and ability to produce offspring (Franke & Fischer 2013). Similar effects were

seen in a large, natural butterfly metapopulation with negative impacts on larval survival, adult longevity, and egg-hatching rate (Saccheri et al. 1998).

Selecting multiple source populations for reintroduction can increase gene pool variability and avoid inbreeding, but it is important to consider whether individual sources are sufficiently similar to be able to mate and avoid outbreeding depression (McClelland and Naish 2007), which is a reduction in fitness due to mating of highly dissimilar genotypes (Lynch 1991). Inbreeding depression and outbreeding depression can have equally damaging effects on the health of populations (Edmands 2007). However, outbreeding has been found to be less likely to occur than has been historically predicted (Frankham et al. 2017; Weeks et al. 2017; Kronenberger et al. 2018). A review of empirical data indicated that most matings among individuals from distant populations failed to develop outbreeding depression, even if those populations had been isolated for thousands of generations (Frankham et al. 2011). The probability of outbreeding depression is elevated when populations exhibit at least one of the following: mating occurs between two distinct species, the populations have fixed chromosomal differences, have not exchanged genes in the last 500 years, or inhabit very different environments. While the potential for inbreeding depression might be higher for most species, the effects of both inbreeding and outbreeding can be detrimental and therefore it is important to consider both.

1.3.1.2 *Genetic considerations: Match to the local environment*

Differences in habitat conditions can exert selection pressures that result in genotypes specifically adapted to the local landscape and climate, a condition known as local adaptation (Savolainen et al. 2007). Local adaptation of populations is an important consideration for population reintroduction as it may limit success of reintroduced individuals if environmental conditions in the reintroduction site differ from those experienced by the source population (Lesica and Allendorf 1999). The adaptive potential and pre-existing adaptation strategies provide two approaches to using genetic information to increase the chance that an introduced population will be suited to conditions of the proposed reintroduction site (Houde et al. 2015). The adaptive potential strategy builds upon the theory that the rate of adaptation in a population is proportional to the genetic diversity (Houde et al. 2015); this strategy aims to maximize genetic diversity of the reintroduced population, by selecting diverse founders, to facilitate adaptation to the new environment. In contrast, the pre-existing adaptation strategy aims to maximize the initial local adaptation of the reintroduced population by selecting founders from a source population that experiences environmental conditions matching the reintroduction site.

There is some debate regarding which of these two strategies is more effective. A comprehensive review recommends that the pre-existing adaptation strategy be considered first, as even if a founding group has high genetic diversity, individuals in that group will likely have low fitness if they are not pre-adapted to the new environment (Houde et al. 2015). However, this strategy is specifically recommended, and most effective, when key environment features affecting individual fitness have been

identified. In cases where the key environment and habitat features affecting individual fitness have not yet been clearly identified, the adaptive potential strategy may be the most practical option. Selecting founders to maximize adaptive potential will also be beneficial if environmental conditions at the reintroduction site change over time (e.g., due to climate change).

1.4 Mottled Duskywing

The Mottled Duskywing *Erynnis martialis* (Scudder, 1870) (Lepidoptera: *Hesperiidae* Figure 1-1) is a medium-sized, brown coloured butterfly belonging to the skipper subfamily Pyrginae, and is endemic to North America (COSEWIC 2015). Adults fly in the summer, in one or two separate broods depending on geographic range. Following fertilization, female butterflies lay their eggs on one of two larval food plant species, New Jersey Tea (*Ceanothus americanus*) and Prairie Red Root (*Ceanothus herbaceus*) (COSEWIC 2015; Figure 1-2). The larvae feed on the leaves of these plants and following maturation, either pupate and emerge as butterflies where there is a second brood or overwinter as a mature larva in a winter leaf nest before pupating and emerging the next spring. Mottled Duskywings are believed to disperse less than other Duskywing species based on their localized population distribution and anecdotal information from naturalist observations, although little information exists on dispersal ability and distances (Burke et al. 2011). Predicted habitat requirements based on field observations outlined in the recovery report for the species includes presence of the host plant(s) (Olson 2002) in multiple patches (Schweitzer et al. 2011), presence of nectar plants (COSEWIC 2015), partial shade (Olson 2002), moist soil (Schweitzer et al. 2011), and

variable land topography (Scott 1986). Mottled Duskywing are opportunistic generalists regarding nectar plant preferences and have been observed in the field to be frequently nectaring on their larval host plants, as well as Oxeye Daisy, Red Clover, Canada Anemone, Wild Columbine and more (Natural Resource Solutions Incorporated, unpublished data).

Figure 1-1. Photo of a Mottled Duskywing (*Erynnis martialis*) in the wild. Photo credit: Shayla Kroeze.

Figure 1-2. Mottled Duskywing host plants, New Jersey Tea (Figure A; *Ceanothus americanus*; photo credit: Shayla Kroeze) and Prairie Red Root (Figure B; *Ceanothus herbaceus*; photo credit: Bob Bell).

The Mottled Duskywing was listed as endangered by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) in 2012 (COSEWIC 2015) and is the only species of butterfly listed as endangered by the Committee on the Status of Species at Risk in Ontario (COSSARO) under the Ontario Endangered Species Act (ESA; Ontario Provincial Government 2022). It is restricted to a few small, isolated subpopulations where its larval host plants occur (COSEWIC 2015; Figure 1-3). Historically in Canada, the Mottled Duskywing was found in southeastern Manitoba, southern Ontario, and southwestern Quebec. The species was last observed in Quebec in the 1950s and is thought to be extirpated from the province (COSEWIC 2012). Additionally, severe population declines have occurred in the United States (COSEWIC 2015).

Currently in Ontario, known persisting populations are in Burlington, Roseneath, Centreton, and Marmora (Figure 1-3). All sites where the species is found are variable in their environmental topography and characteristics. In addition, small numbers of Mottled Duskywing have been observed in Camp Borden, Oakville, and other locations in the Rice Lake Plains. Threats to the Mottled Duskywing include loss of habitat, pesticide use, host plant competition with invasive species, and climate change (COSEWIC 2015). Limitations to recovery include the Mottled Duskywing's high degree of habitat specificity, predicted low dispersal ability (COSEWIC 2015), and shifting phenology caused by climate change (Kingsford and Watson 2011).

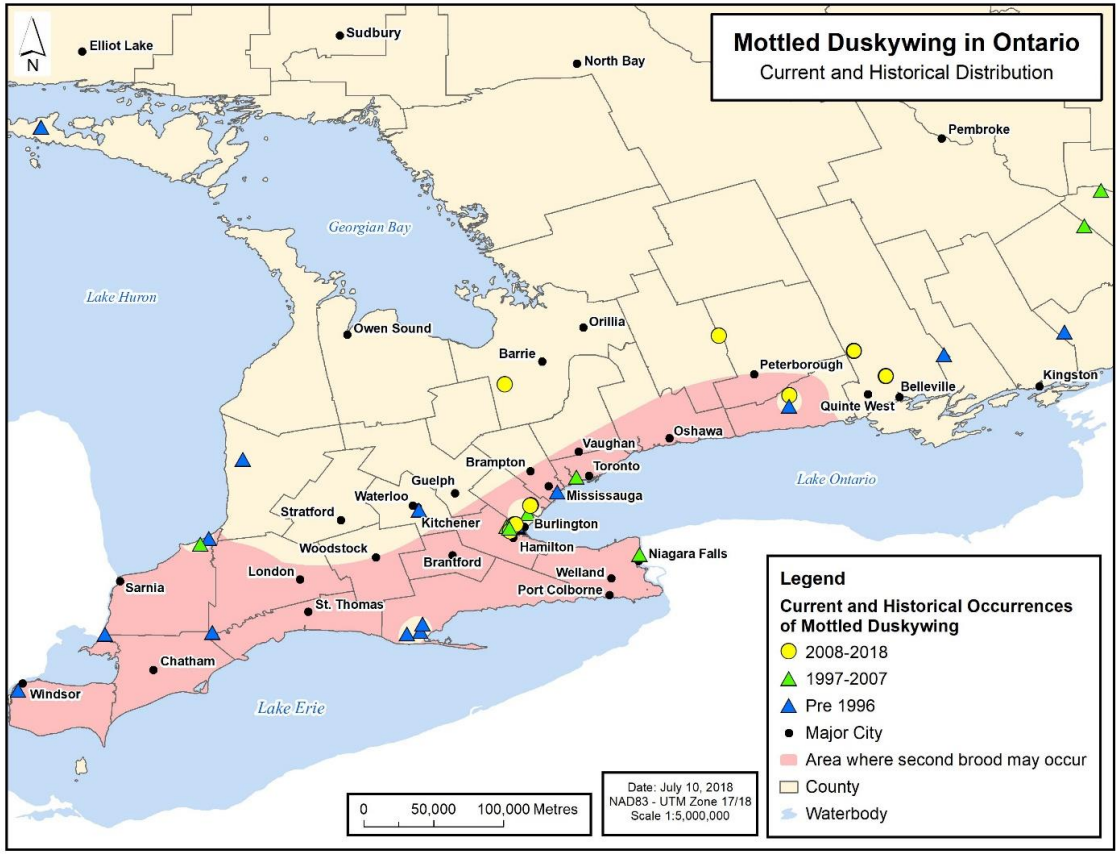

Figure 1-3. Current and historical distribution of the Mottled Duskywing (*Erynnis martialis*) in Ontario. Originally published in COSEWIC 2015, updated in 2016 by Natural Resources Solutions Incorporated. Permission to include from Jessica Linton (NRSI).

1.4.1 Reintroduction efforts for Mottled Duskywing

As part of the recommended recovery plan, a collaborative effort began in 2017 to release Mottled Duskywing individuals in Pinery Provincial Park, where the species historically occurred. That reintroduction has been initiated, with the first founders having been introduced into Pinery in the summer of 2021, and was associated with a mark-recapture program, captive breeding, and this genetic research. This work was authorized by the Ministry of Environment, Conservation and Parks through a permit under the Endangered Species Act. This first reintroduction site may eventually be followed by reintroduction to other locations in Ontario, including one tentatively planned in Norfolk County, as well as augmentation of currently extant but small populations. Conservation of the Mottled Duskywing is not only beneficial for the species, but also promotes protection of globally rare oak-savanna ecosystems, as well as tallgrass prairie communities, and the many species that utilize these habitat types at Pinery Provincial Park (Marotta 2021).

1.5 Thesis objectives

My research aimed to support and inform conservation reintroduction efforts for the Mottled Duskywing by:

1. Developing microsatellite loci for *Erynnis martialis* that can be used for populations genetic analyses (Chapter 2).
2. Characterizing genetic diversity among extant populations of Mottled Duskywing in Ontario, as well as known populations in Manitoba, New York, and Michigan

to assess if populations are sufficiently diverse to support a reintroduction (Chapter 3).

3. Characterizing genetic differentiation among populations to better understand pattens of gene flow and connectivity, provide insight into potential dispersal ability, and assess the potential mixing of populations for reintroduction sourcing (Chapter 3).
4. Estimating effective population sizes and testing for evidence of recent bottlenecks in extant populations to provide new insight into demographic stability (Chapter 3).

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Chapter 2

2 Development of tetranucleotide and dinucleotide microsatellites for the at-risk Mottled Duskywing butterfly, *Erynnis martialis*

2.1 Abstract

Twenty-four microsatellite loci for an at-risk North American butterfly, the Mottled Duskywing (*Erynnis martialis*), were isolated and characterized. Forty-one tetra- and di-nucleotide loci were originally identified and selected using Illumina next-generation sequencing, and these were refined to 24 variable and cleanly amplifiable loci using fragment analysis. I also describe conditions by which these microsatellites can be multiplexed in eight separate reactions. These microsatellite loci were developed to investigate population differentiation and diversity of the Mottled Duskywing in Ontario, Canada where the species is listed as endangered. The microsatellites will be used to inform reintroduction and conservation protocols for the species in Ontario. The number of observed alleles at the 24 microsatellites in a sample of 34 Mottled Duskywing from a single location ranged from 2 to 10 with observed levels of heterozygosity ranging from 0.06 to 0.76 and expected levels of heterozygosity ranging from 0.06 to 0.79.

2.2 Body

Erynnis martialis (Scudder, 1870), commonly known as the Mottled Duskywing, is a medium-sized, brown coloured butterfly belonging to the skipper family (Lepidoptera: *Hesperiidae*; *Pyrginae*; COSEWIC 2015). It is endemic to North America, and due to threats, such as loss of habitat, pesticide use, host plant competition with

invasive species, and climate change, in the province of Ontario, Canada the species is now only represented by a limited number of small, isolated populations (COSEWIC 2015). It is listed as endangered in this province by the Committee on the Status of Endangered Wildlife in Canada. The only previously published genetic information on the Mottled Duskywing consists of DNA barcodes (COSEWIC 2012).

Microsatellites were developed to characterize genetic diversity and differentiation of extant populations and inform planned reintroductions of the species to locations from which it has been extirpated. Previous research shows that it is critical to consider genetic structure and diversity of a species to optimize strategies for reintroduction programs (Daniels et al. 2018), particularly for selection of a source population (Drauch et al. 2008). Microsatellites were selected because of their co-dominance and high variability (Regnaut et al. 2006; Chassaing et al. 2018), particularly given that a limited number of individual butterflies were initially available for marker development (12 female butterflies from a single population, with a 2019 population size estimate of 252-513 individuals; Demarse, unpublished data).

Genomic DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD) from 12 whole, mated female butterflies provided by a simultaneous captive rearing program located at the Cambridge Butterfly Conservatory (Ontario, Canada). A sterile pestle was used to manually crush and grind tissue from the head, thorax, and legs of each individual; the abdomen was excluded to avoid risk of including DNA from sperm. A single elution with 200 µl of MilliQ water warmed to 36 °C was used to collect the DNA and a Qubit fluorometer (Invitrogen) was used to quantify the

DNA. An equimolar DNA pool from all twelve individuals (105.84 µL total volume) was sent to GenoScreen Services (Lille, France; www.genoscreen.fr), where the DNA pool was enriched for microsatellite loci and subsequently sequenced using their Geno Sat® service. In brief, a microsatellite-enriched genomic library was developed and sequenced using the Illumina MiSeq platform to generate 250 bp paired-end reads following manufacturer guidelines from MiSeq Reagent Nano Kit v2. Approximately 1.30 million reads were obtained and merged into 590,832 contigs with PrinSeq software. The bioinformatics program QDD v3 (Meglécz et al. 2014) was used to detect and select microsatellites and design primers. A total of 836 primer pairs were returned and of these, I tested 41 for polymorphism and consistency in scoring. Only perfect di- and tetra-nucleotide repeats were considered to avoid potential introduction of stop codons within reading frames, and to increase the probability of accurate scoring.

Twenty-two additional Mottled Duskywings were sampled non-lethally in Marmora, Ontario, Canada; this is the same population from which the 12 females used to identify the microsatellites originated. DNA for each of these 22 individuals was extracted from small, non-lethally sampled pieces of wing tissue (Koscinski et al. 2011) using the Qiagen DNeasy Blood and Tissue kit, with a 200 µL final elution with Milli Q water warmed to 36 °C. Both the non-lethal samples and the whole females were collected in the same year (2019), and the genotype data from all 34 individuals was combined to characterize variation at the microsatellite loci.

Each of the 41 selected di- and tetra-nucleotide microsatellites were initially amplified in a sub-set of two to three individuals in a 10 µl PCR reaction containing

forward and reverse primers (0.2 µM each), buffer (1X), $MgCl_2$ (3 mM), BSA (0.15 mg/ml), dNTPs (0.2 mM), AmpliTaq DNA polymerase (0.25 U), and DNA template. The thermal cycling parameters used for amplification were: 96 °C for 5 min; 30 cycles of denaturation at 96 °C for 30 s, annealing at various temperatures for 30 s, and elongation at 72 °C for 60 s; final extension at 72 °C for 5 min. The optimized annealing temperature varied by locus as designated (Table 2-1).

Table 2-1. Characterization of 24 microsatellite loci in the Mottled Duskywing (*Erynnis martialis*). N_R is the range of the number of repeats, T_m is the annealing temperature in °C, *N* is the number of individuals successfully genotyped (out of 34), bp is the size range of the microsatellite in base pairs, N_a is the number of unique alleles observed, H_O is the observed heterozygosity, and H_E is the expected heterozygosity.

Locus	Repeat Unit	Primer sequence (5′–3′)	N_R	T_m	*N*	bp	N_a	H_O	H_E
EMusat2	ACAT	F: TCAGCTTTATGTCCGGGCAA	39–48	54	29	157–193	4	0.62	0.53
		R: GCCACTACTTGCGACTAATGAG							
EMusat3	AAAT	F: TGGACTGGTGTACCGAAAGT	33–46	54	27	132–184	5	0.47	0.56
		R: TCCTTTCCGTTATATCAAGAAGCG							
EMusat4	AAAG	F: TCAGTAAGGCAAGACTGTTAGCA	64–74	54	34	254–294	9	0.76	0.76
		R: AGTATGGATTAGAGTATCGAGGTCT							
EMusat5	AATT	F: TCTTGATCTAACGTAGTATCGTACG	56–58	53	31	224–232	2	0.62	0.41
		R: TCTACGCAATGCATCAATATTTGA							
EMusat8	AAAC	F: CGCCGGTACTGACAGCTAAA	61–80	55	33	245–321	8	0.62	0.68
		R: GCGAGCGAAATGAGTCCAGA							
EMusat9	ACCT	F: CTGAAGGTGTATCACAGGCAGT	61–68	55	34	243–271	3	0.65	0.62
		R: ACTCTTAACGTCGCGTGTCT							
EMusat10	AACG	F: AGGAAATCGTTTGCCAAATACGT	48–52	54	27	192–208	5	0.35	0.56
		R: TGTACCCTCTATCACAACGAATG							
EMusat11	AAGG	F: AGACGTATTGGATATTTGGAATGAGT	48–49	54	33	191–195	2	0.21	0.32
		R: TGCTAGGACAAGAGCCGATT							
EMusat12	AAAC	F: TCAGAGAAACATACTCGACAATGC	44–49	54	34	175–195	2	0.62	0.50
		R: TGGCCGTTAGCTCACATCTC							
EMusat13	ACAT	F: ACCAGAGTGGAGATTTGACTACC	35–49	55	32	139–193	3	0.26	0.44
		R: GGGACTCGCCGTACTAAGC							
EMusat14	ACAT	F: AGTAGGTTAAACAGGGCGAACC	69–72	55	34	275–287	4	0.41	0.50
		R: GCCGCAGTCAGAGACATAGG							
EMusat16	ACAG	F: GCCAACATCCTTCTCGTTGC	62–66	55	34	247–263	3	0.38	0.38
		R: TCATGTCATATGCTTCGTTTGGG							
EMusat17	AAAT	F: CCTACCATGGGACCAGTAAGC	58–59	54	34	231–235	2	0.53	0.50
		R: GCAATCGTTAAACTTTATCAGAGGC							
EMusat18	AAAC	F: TCCAAACTTGACACCGAGAA	48–60	52	34	191–239	6	0.50	0.59
		R: TGGCCTTTATTGTCATTCAATTGT							
EMusat20	AAAT	F: TGGCAAAGGTCACTTACGCT	40–44	55	30	160–176	5	0.62	0.56
		R: ACTGGTATGTCCATGGCACC							
EMusat22	AACT	F: ATGGTTCACGGAAGGACCTG	32–39	54	33	126–154	4	0.47	0.59
		R: TGAATACCAAATCGAAGACTGACT							
EMusat25	AAAT	F: CCACGACACAGGGAATCCTA	30–31	54	34	121–125	2	0.26	0.26
		R: GGCTATGACTTCCCGGGTTC							
EMusat27	AAAT	F: CCTGCCATGGGACCAGAAAG	27–28	54	32	107–111	2	0.06	0.06
		R: ACATTTGATGCACCTTATAATATGAGT							
EMusat31	AG	F: AGTGTGGATTCAGAAGGCGC	89–97	55	32	178–194	6	0.38	0.41
		R: CACGTTAACTGTCGGGCAAA							
EMusat34	AG	F: GGGTCGGGTCTCCTCATACT	100–114	55	34	200–228	7	0.41	0.56
		R: CCTAGAGTGCACAACTGAGCA							
EMusat35	AC	F: ACTGAGGCCTAGACCCTAGC	112–117	55	33	223–233	5	0.32	0.68
		R: ATTGAGGCCTTCAGACTGCC							
EMusat36	AG	F: GTCCAGTATCTCAGCAGACGG	115–128	55	34	230–256	6	0.44	0.68
		R: AAGGAAGAACAGTGCTAACGCT							
EMusat37	AG	F: ACAGTCTACTAGCCATTCCGT	62–68	54	34	123–135	5	0.56	0.74
		R: TGACCATGACAGCTGTACCAT							
EMusat40	AG	F: AGGCAAATTACAATCAAAGCATCA	139–145	54	34	278–290	6	0.71	0.79
		R: GTGAGCAGTTCGTCGTTTCC							

PCR products were electrophoresed and visualized on agarose gels to check for clean amplification and the presence of bands of the expected sizes. Loci that met these criteria were then amplified using one fluorescently labelled primer per pair (6-FAM, VIC, NED, or PET label), and sized by fragment analysis on a 3730S capillary DNA analyzer, using LIZ-500 size standard (Applied Biosystems, Foster City, CA), at the University of British Columbia's Sequencing and Bioinformatics Consortium (British Columbia, Canada). Genotypes were called using GeneMarker® HID software (SoftGenetics, State College, Pennsylvania, USA). Loci that did not amplify cleanly (i.e., had more than two peaks), or could not be consistently called were removed from further consideration. This left me with 24 loci that I characterized in the full set of 34 individuals. Three loci (EMusat10, EMusat13, EMusat35) showed either significant deviation ($P < 0.05$) from Hardy Weinberg equilibrium according to analysis using genepop'007 (Rousset 2008) or evidence of null alleles using MICROCHECKER v2.2.3 (Van Oosterhout et al., 2004).

Finally, multiplexes were created to optimize the number of loci that could be simultaneously amplified, by varying the concentrations of primers, as summarized in Table 2-2. I was able to successfully multiplex 18 of the 24 selected loci in six separate reactions.

Table 2-2. Final primer concentrations for PCR amplification of Mottled Duskywing microsatellites including multiplex PCRs. FD refers to the fluorescent dye used to identify each microsatellite, Amp refers to whether the locus amplifies in a multiplex with multiple primers or as a single locus, and Final concentration refers to the final concentration of the corresponding primers in the PCR.

Set	Locus	FD	Amp	Final concentration
Set 1	EMusat14	NED	Multi	0.2 µM
	EMusat16	PET	Multi	0.2 µM
	EMusat8	VIC	Multi	0.2 µM
	EMusat9	6-FAM	Multi	0.2 µM
	EMusat25	VIC	Single	0.2 µM
Set 2	EMusat36	NED	Multi	0.4 µM
	EMusat35	6-FAM	Multi	0.1 µM
	EMusat34	VIC	Multi	0.2 µM
	EMusat22	NED	Single	0.12 µM
Set 3	EMusat40	NED	Multi	0.2 µM
	EMusat4	PET	Multi	1 µM
	EMusat17	VIC	Multi	0.2 µM
	EMusat10	6-FAM	Multi	0.1 µM
	EMusat37	NED	Multi	0.2 µM
	EMusat27	6-FAM	Multi	0.1 µM
Set 4	EMusat18	VIC	Single	0.3 µM
Set 5	EMusat31	NED	Multi	0.2 µM
	EMusat13	PET	Multi	0.8 µM
	EMusat11	6-FAM	Single	0.06 µM
Set 6	EMusat12	NED	Multi	0.2 µM
	EMusat2	PET	Multi	0.4 µM
	EMusat3	VIC	Multi	0.2 µM
Set 7	EMusat20	NED	Single	0.3 µM
Set 8	EMusat5	6-FAM	Single	0.3 µM

This set of loci will be a valuable tool for informing conservation of the Mottled Duskywing, including protocols for reintroductions of the species, augmentation of small populations, and assessment and monitoring of genetic diversity and structure in current and introduced populations.

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Chapter 3

3 Population genetic analysis of the at-risk Mottled Duskywing (*Erynnis martialis*) to inform species reintroductions

3.1 Abstract

Species reintroduction or translocation programs are increasingly used to reverse global declines in biodiversity. Information on genetic diversity and structure within a species can be critical for successful selection of a source population for such programs. The Mottled Duskywing (*Erynnis martialis*) is a North American butterfly listed as endangered in Ontario, Canada, and a collaborative effort to reintroduce the species has begun. Using 21 microsatellite markers, I evaluated metrics of genetic diversity, population structure, and demographic stability of Mottled Duskywing populations in Ontario, Manitoba, and neighbouring U.S. States. These data were used to inform the selection of a source population for an initial reintroduction to Pinery Provincial Park in Grand Bend, Ontario. Mottled Duskywing populations had moderate levels of genetic diversity. Additionally, populations in close proximity had similar allele frequencies, while populations more than 8 km apart had high average genetic differentiation suggesting limited gene flow. Therefore, future management plans might consider populations within an 8 km range as a single management unit. These data can be used to inform future reintroductions or population augmentations of the species, and to assess genetic status of both remnant and reintroduced populations.

3.2 Introduction

Fragmentation, degradation, and loss of suitable habitat coupled with climate change due to growing human populations have caused dramatic loss of global biodiversity (Oliver and Morecroft 2014). It has become necessary to develop effective strategies for the conservation of declining and endangered species (Bainbridge 2014). Several strategies have been implemented to offset this loss of biodiversity including regulations on harmful practices such as overharvesting or release of pollutants, landscape management, invasive species management, and protection of critical habitats (Apollonio et al. 2010; Deinet et al. 2013; Brown 2013). Another strategy that has emerged, often as a final attempt at maintaining or restoring biodiversity in the wild, is species translocation or reintroduction (Seddon et al. 2007; Attard et al. 2016).

The goal of reintroduction is to establish a new population which is self-sustaining and has a low probability of extinction (Fraser 2008). Previous research on reintroduction programs shows that it is critical to consider genetic structure and diversity of a species to optimize strategies for implementation of such programs (Attard et al. 2016, Daniels et al. 2018). Knowledge of the genetic variation within a species is important in conservation for a variety of reasons. Such information helps to define management units and informs the preservation of genetically distinct populations (Haig 1988; Hedrick 2001). Genetic information can provide insights into past changes in population size, connectivity among different populations, potential for inbreeding, and other factors that may contribute to extinction risk through genetic or demographic stochasticity (Yang et al. 2018; Groenen et al. 2012; Lowe and Allendorf 2017). Another

important way in which genetic data can inform conservation is through the selection of an appropriate source population or populations for reintroductions (Drauch et al. 2008).

If founders for a new population are chosen from a source population that is small, or too few individuals are used for reintroduction, the new population will have low genetic diversity and a potentially low effective population size (Tracy et al. 2011). Low diversity in the introduced population in turn results in a risk of inbreeding depression and may also lessen the ability of the population to adapt to changing future environmental conditions (Hughes and Sawby 2004). Selecting multiple source populations for reintroduction can increase gene pool variability and improve likelihood of avoiding inbreeding. However, mixing source populations carries its own risks. One such risk is mating incompatibility, which can be caused by pre- or post-zygotic reproductive barriers or by the presence of reproductive parasites such as *Wolbachia* (Werren et al. 2008). *Wolbachia* leads to mating barriers through different reproductive phenotypes resulting from infection, the most common being cytoplasmic incompatibility where the sperm and eggs are not able to produce viable offspring if mating individuals do not have the same infection status (Werren et al. 2008). A second risk of mixing source populations is outbreeding depression (Lynch 1991, McClelland and Naish 2007), which is a reduction in fitness of the offspring of highly divergent genotypes (Lynch 1991), caused by disruption of co-adapted gene complexes (Orr 1996) and loss of local adaptation (Templeton et al. 1986).

In addition to inbreeding avoidance and longer-term evolutionary potential, the degree of adaptation of the initial founders to the new environment is a critical concern in

reintroductions. If early generations are poorly adapted to the new, local environment the populations may never become successfully established. One strategy to address this issue that also addresses risks of inbreeding and longer-term adaptation to changing conditions, is the adaptive potential strategy (Houde et al. 2015). This strategy aims to maximize genetic diversity among founders, such that the new population can rapidly adapt to conditions in the site of reintroduction. Alternatively, founders can be selected that are pre-adapted to, and have high fitness in, the reintroduction site; two strategies to achieve this outcome are ancestry matching and environment matching (Houde et al. 2015). In ancestry matching, a source population is selected that is most genetically similar to the extirpated population at the designated reintroduction location. This strategy requires information on the genetic make-up of the historic, extirpated populations. In environment matching, a source population is selected that occupies an environment most similar to that of the reintroduction location. This strategy requires detailed information on the habitat and environmental characteristics of both the reintroduction location and locations of potential source populations, as well as strong understanding of the key factors affecting survival and reproduction of individuals.

Butterflies are one of the insect taxa in which species declines have been widely documented (Sánchez-Bayo and Wyckhuys 2019), and many attempts have been made to reintroduce various butterfly species (Nakahama et al. 2022; Davis et al. 2021; Dincă et al 2018; Andersen et al. 2014; Soorae 2010; Wynhoff 1998). Very few earlier butterfly reintroductions were successful, which has been attributed to a lack of accepted guidelines or failure to follow recommended protocols, such as the recommendation to release >60 individuals sourced form a large heterogenous population to maintain genetic

diversity and thorough assessment of reintroduction habitat (Joyce and Pullin 2003). Failure of the reintroduction of the Miami Blue Butterfly (*Cyclargus thomasi bethunebakeri*), for example, is attributed to limited reintroduction sites, difficulty rearing larvae, and tropical cyclones at the reintroduction sites (Soorae 2010). In contrast, successful reintroduction of the Large Blue Butterfly (*Maculinea arion*) in England has been largely attributed to strict adherence to the reintroduction guidelines of the International Union for the Conservation of Nature (IUCN/SSC 2013). Indeed, 19 generations after reintroduction, Andersen et al. found the introduced population showed no loss of genetic diversity even though it represented a unique subset of genetic diversity compared to its source population (2014).

The Mottled Duskywing, *Erynnis martialis* (Scudder, 1870) is a medium-sized, brown butterfly belonging to the skipper family (Lepidoptera: *Hesperiidae*; *Pyrginae*; COSEWIC 2015). It is endemic to North America where it has become uncommon to rare. It was listed as endangered in Ontario, Canada in 2012, with threats to the populations including loss of habitat, pesticide use, host plant competition with invasive species, and climate change (COSEWIC 2015). Severe population declines have also been observed in the United States, where the species has become extirpated in some regions (COSEWIC 2012). As a part of the recovery plan for the Mottled Duskywing in Canada, the species is being reintroduced to Pinery Provincial Park in Grand Bend, Ontario, where the species historically occurred but has since been extirpated. Future reintroduction to Norfolk County is also being considered.

Though historical Mottled Duskywing specimens from the reintroduction site were available, I was not able to successfully extract DNA extraction from them. Therefore, the genetics of that historical population are unknown, and the adaptive potential strategy could not be considered. Additionally, no research to date aside from field observations has been published regarding environmental preferences of *Erynnis martialis*, except for presence of its host plants. Therefore, I focused on examining the genetic diversity and structure, as well as demographic stability, of the Mottled Duskywing populations in Ontario, Canada, to inform selection of a source population using the adaptative potential strategy. To analyze genetic structure and variation in the species more broadly, my analyses also included some of the nearest populations in the United States (NY, MI) as well as the other remaining Canadian populations in the province of Manitoba. Data from 21 polymorphic microsatellite markers were used to assess genetic diversity of potential source populations to evaluate different reintroduction scenarios. Additionally, I assessed genetic divergence and genetic structure among populations, as well as demographic stability of the populations through estimation of effective population sizes and bottlenecks, to inform Mottled Duskywing biology and conservation, more generally.

3.3 Methods

3.3.1 Sample collection

Twelve whole female adult Mottled Duskywing butterflies, originally from Marmora, Ontario were obtained from a captive rearing experiment at the Cambridge Butterfly Conservatory, after they had died in captivity. Upon their deaths and following arrival in the laboratory, they were stored in a -80 °C freezer.

In 2019, I collected non-lethal wing or leg samples from most known extant populations in Ontario, Canada including Burlington, Marmora, Oakville, and the Rice Lake Area (Figure 3-1). A population in Northern Michigan was also sampled in 2019. In 2020, I resampled some of these sites to assess temporal variation in allele frequencies and estimate genetic drift and obtained samples from additional sites in the Rice Lake and Burlington areas in Ontario, as well as in eastern New York State, and sites from southern Manitoba. I defined different populations as sites inhabited by *E. martialis* that are 2 km or farther apart; without prior knowledge on genetic variation in the species it was possible that some of the samples did not represent truly isolated populations, but this would be revealed by my analyses (Figure 3-1).

In total, 312 Mottled Duskywings were sampled (Table 3-1). At each site, butterflies were captured by netting and placed in a small jar. The jar was placed on ice in a cooler for approximately 15 min to calm the butterfly. After 15 min of cooling, the butterfly was removed from the jar and either a small (approximately 0.25 cm^2) sample of wing tissue from each hind wing or a single hind leg was collected from each butterfly using forceps. After each individual was sampled, forceps were cleaned by wiping with

70 % isopropyl alcohol swabs before re-use. Wing and leg samples were immediately placed individually in Eppendorf tubes filled with absolute ethanol or filled with silica powder covered with a thin layer of cotton batting to avoid silica sticking to butterfly tissue. Lastly, prior to release, butterflies were marked with a small amount of acrylic paint on either the wing or the thorax to avoid re-sampling. Though no published information exists regarding longevity of paint marks in the field, individuals have been observed with paint marks up to 21 days after marking (Demarse, unpublished data). Upon arrival at the laboratory, all samples were stored at -80 °C. A maximum of 30 butterflies were sampled from each site per year to minimize negative impacts to at-risk populations. Removal of these 'wing clips' mimics regular wear and tear that would occur on the wings of a butterfly. Extensive research has shown that this method does not affect a butterfly's ability to fly, survive, or mate (Crawford et al. 2013; Hamm et al. 2010; Figure 3-2). Similarly, removal of a leg has been demonstrated to have no measurable effect on survival or behaviour of butterflies, even small, delicate species (Marschalek et al. 2013).

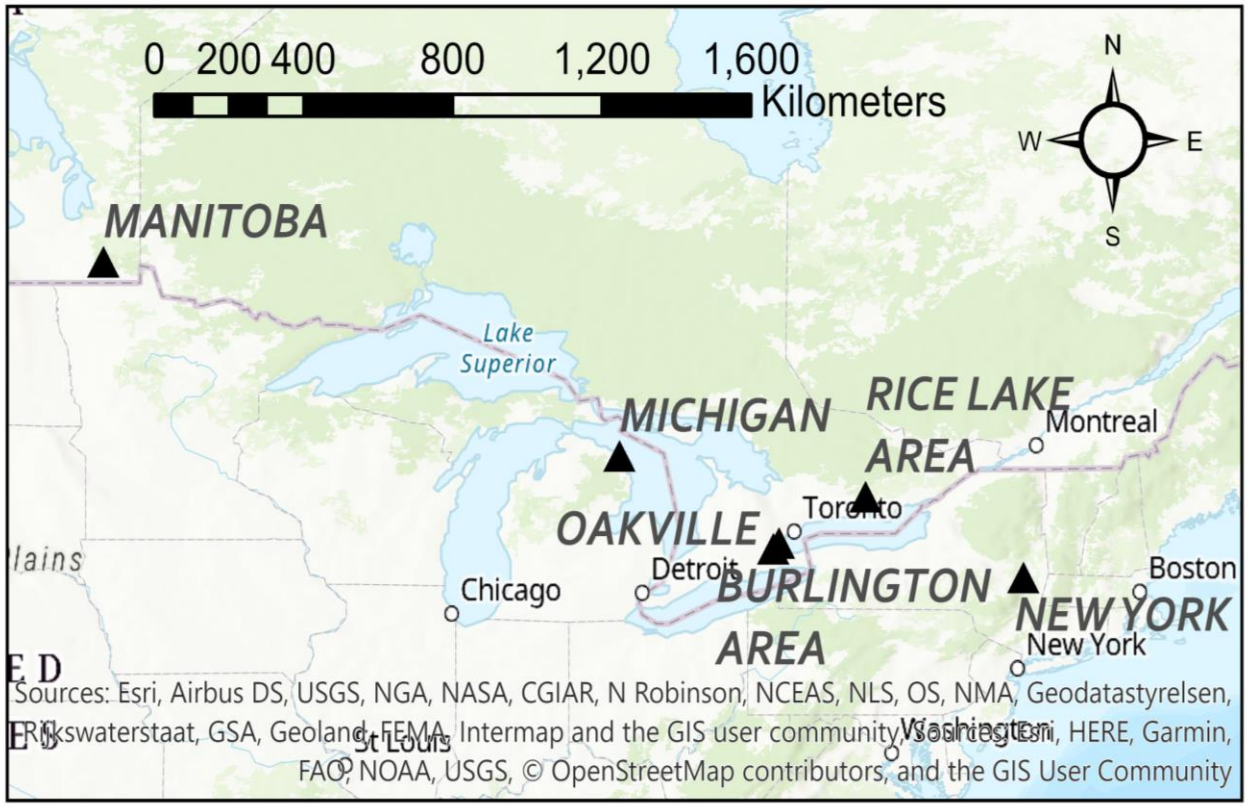

Figure 3-1. Locations of Mottled Duskywing populations sampled for population genetics analyses. Only general areas disclosed due to sensitivity of this information, considering the endangered status of the species. Created using ArcGIS Pro (ArcGIS 2010).

Table 3-1. Breakdown of Mottled Duskywing samples collected from all known populations with permission to sample from Ontario, southern Manitoba, eastern New York, and northeastern Michigan. Whole butterfly samples provided by the captive rearing program and non-lethal samples refer to wing clips or a leg collected in the field. Latitude and longitude, and more specific locations are not reported due to the sensitivity of this information given the endangered status of the species.

Site	Province or State	2019 whole butterflies	2019 non-lethal sample	2020 non-lethal sample	**Total**
Burlington	Ontario	0	26	30	**56**
Burlington 2	Ontario	0	5	0	**5**
Burlington 3	Ontario	0	0	2	**2**
Burlington Gas Line	Ontario	0	5	30	**35**
Burlington Hydro Corridor	Ontario	0	5	0	**5**
Manitoba	Manitoba	0	0	17	**17**
Marmora	Ontario	12	18	30	**60**
Michigan	Michigan	0	11	12	**23**
New York	New York	0	0	30	**30**
Oakville	Ontario	0	1	15	**16**
Rice Lake 1	Ontario	0	13	30	**43**
Rice Lake 2	Ontario	0	0	10	**10**
Rice Lake 3	Ontario	0	0	10	**10**
Total		**12**	**84**	**216**	**312**

Figure 3-2. An unsampled Mottled Duskywing in the wild that has experienced natural wing damage. The natural damage is similar in size and shape to the damage resulting from collection of a non-lethal wing clip removed for DNA extraction. Photo credit: John Christensen.

3.3.2 DNA Extraction

Genomic DNA was extracted with a DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD), using a sterile pestle to crush and grind butterfly tissue for better lysis of cells. Extraction methods from whole butterflies are outlined in Chapter 2. For wing clips and legs, the entire sample was used. A single elution with 200 µl AE buffer warmed to 36 °C was used to collect the DNA.

3.3.3 Microsatellite genotyping

Primers and multi-plex PCR protocols described in Chapter 2 were used to amplify microsatellites from all samples. All individuals were genotyped at a total of 24 loci that amplified cleanly and consistently and were variable (see Chapter 2). Microsatellite loci were amplified using one fluorescently labelled primer per pair (6-FAM, VIC, NED, or PET label), and sized by fragment analysis on a 3730S capillary DNA analyzer, using LIZ-500 size standard (Applied Biosystems, Foster City, CA), at the University of British Columbia's Sequencing and Bioinformatics Consortium (British Columbia, Canada). Genotypes were called using GeneMarker® HID software (SoftGenetics, State College, Pennsylvania, USA).

3.3.4 Data analysis

Samples taken from the same location in different years were analyzed separately as unique populations. All data analyses only included samples where more than 5 individual butterflies were sampled in a given year. All loci were checked for deviations from Hardy Weinberg Equilibrium (HWE) or for evidence of linkage disequilibrium, within each population, using GENEPOP'007 software (Rousset 2008). Additionally, loci

were checked for evidence of null alleles using MICROCHECKER v2.2.3 (Van Oosterhout et al., 2004).

3.3.4.1 *Genetic diversity*

As metrics of genetic diversity, I calculated expected heterozygosity (H_E; the proportion of individuals in a population that are expected to be heterozygotes under HWE) and observed heterozygosity (H_O; the proportion of heterozygous loci across all loci in all individuals), for each sample, using R statistical software (R Core Team 2020) with packages 'adegenet' (Jombart 2008), 'pegas' (Paradis 2010), and 'hierfstat' (Goudet 2005). Expected heterozygosity is calculated using the equation $1 - \sum p_i^2$, where p_i is the frequency of the *i*th allele, and the operator is summation over all alleles (Jombart 2014). Additionally, I calculated allelic richness (A_R; number of alleles independent of sample size), using R statistical software (R Core Team 2020) and the package 'diveRsity' (Keenan et al. 2013). Allelic richness is calculated in this package using 1000 re-samples where n is the smallest sample in the input data file, with replacement per locus per population sample (Keenan 2017).

3.3.4.2 *Population structure*

I calculated Fixation Index (F_{ST}; measure of population differentiation due to genetic structure; Weir and Cockerham 1984) using GENEPOP'007 software (Rousset 2008) for all population pairs. For sites where there were sufficient sample sizes for both 2019 and 2020 sampling years, only the sampling year with more individuals sampled was included in these analyses. F_{ST} ranges from 0 to 1, where 0 indicates no genetic differences and 1 is the maximum possible amount of genetic differentiation (Hartl and

Clark 1997). However, F_{ST} values also depend on the variability of the markers used and cannot often be compared between studies of different species (Meirmans and Hedrick 2011).

To test for isolation by distance (IBD), I assessed the correlation between pairwise genetic differentiation (F_{ST}) and log base 10 geographic distance (km) using a Mantel test implemented with the 'ecodist' package (Goslee and Urban 2007) in R statistical software (R Core Team 2020). I calculated straight-line geographic distances using Google Earth Pro software and log (base 10) transformed the distances due to the wide range (2.2 km–1867.4 km). Where samples were taken in two different years at a given site, I only included the larger of the two samples in the IBD analysis.

To identify and describe genetic clusters within the Mottled Duskywing, I ran a Discriminant Analysis of Principal Components (DAPC) using the 'adegenet' package (Jombart 2008) in R (R Core Team 2020). I used the *find.clusters* function to detect the number of clusters using K-means associated with the lowest Bayesian Information Criterion (BIC). I used the cross-validation function *xvalDapc* to confirm the number of retained principal components (PCs). Discriminant analyses of principal components is a multivariate method used to identify and describe groups of genetically similar individuals (Jombart et al. 2010). This method provides some benefits not provided by Bayesian clustering methods such as STRUCTURE, such as not relying on assumptions about type of population subdivision. In contrast to other multivariate analyses such as a principal component analysis (PCA), DAPC uses K-means clustering of principal components that focuses on between-group variability, while minimizing within-group

variation (Liu and Zhao 2006). Therefore, a DAPC is a more effective at discrimination of individuals into groups (Jombart et al. 2010). Bayesian Information Criterion (BIC) are used to assess the best supported model or number of clusters. On simulated data, DAPC was found to be as accurate as STRUCTURE in detecting clusters, and better at capturing the structure in more complex population genetic scenarios, such as where there is more subtle hierarchical structure or isolation by distance (Jombart et al. 2010).

Additionally, to infer population structure further I used STRUCTURE 2.3 using the no admixture model and correlated allele frequencies (Pritchard et al. 2000; Falush et al. 2003). Like DAPC, STRUCTURE (Pritchard et al. 2000) aims to assign individuals to genetic clusters and has become one of the most widely used programs to assess genetic stratification (Earl and vonHoldt 2012). It is a model-based Bayesian clustering method where there are "K" populations, and each population is characterized by a set of allele frequencies at each locus (Pritchard et al 2000). Sampled individuals are probabilistically assigned to one or more population(s) depending on if they are admixed, in such a way as to minimize Hardy-Weinberg and linkage disequilibrium within the K populations. There are multiple different methods used to identify the optimal K (number of clusters), the most used being the ΔK method (Evanno et al. 2005). The ΔK method is based on the rate of change in the log probability of data between successive K values. While it is effective at identifying the uppermost level of hierarchical population structure, it can fail to detect finer structure and may not perform well when the sample sizes are uneven (Puechmaille 2016). This led to the alternative estimators: MedMedK, MedMeaK, MaxMedK, and MaxMeaK (Puechmaille 2016). These estimators are based on the count of the number of clusters that are contained in at least one subpopulation and were found

to be more accurate on both even and uneven datasets. STRUCTURE is most accurate when run at multiple replications, and because stochastic simulation is part of the inference, independent analyses of the same data can result in several distinct outcomes (Jakobsson and Rosenberg 2007). The computer program CLUMPP takes membership coefficient matrices from multiple STRUCTURE runs and outputs them permuted so that all replicates have as close a match as possible.

No specific mutation model is assumed in STRUCTURE, and it is appropriate for unlinked microsatellite data (Pritchard et al. 2009). I ran 10 replicates at each estimated population number or K (setting K to vary from 1 to 16), with a burn-in of 100,000 iterations and collection of data of 1,000,000 iterations. The upper limit of estimated population number was set to 16 as there were 14 sampled populations (with sites separated by sampling years) and an upper limit of slightly more than 14 would allow for detection of potential subpopulations within sites. The results from STRUCTURE were then processed in STRUCTURE HARVESTER (Earl and vonHoldt 2012) and STRUCTURE SELECTOR (Li and Liu 2018) and the highest means from MedMeaK, MaxMeaK, MedMedK, and MaxMedK were used to infer the optimal K (Puechmaille 2016). Finally, the output with the optimal selected number of populations (in this case, K = 9) was processed using CLUMPAK for averaging of assignment scores and visualization of STRUCTURE bar plots (Kopelman et al. 2015).

3.3.4.3 *Demographic stability*

I determined estimates of variance effective population sizes (N_e) using the linkage disequilibrium method (Waples and Do 2008), as implemented in NeEstimator V2.1 (Do et al. 2014), for populations with more than 5 individuals sampled for both 2019 and 2020 sampling years, as required by the software using base settings. NeEstimator assumes discreet, nonoverlapping generations (Do et al. 2014). Estimation of contemporary N_e using the linkage disequilibrium (LD) method has become an important tool for conservation genetics and is the most used method (Hollenbeck et al. 2016). This method estimates N_e based on temporal allele frequency difference; as a population decreases in effective size, allele frequencies will change more between generations due to random genetic drift and increased inbreeding (Caballero 1994). The difference in allele frequencies is used to estimate the idealized population size that could produce a change as large as the one observed.

I used the program BOTTLENECK (Cornuet and Luikart 1997) to detect evidence of recent bottlenecks in Mottled Duskywing populations, for sites with sample sizes larger than 15 individuals, as required by the program. This analysis is based on the expectation that by preferentially eliminating rare alleles, a bottleneck reduces allelic diversity more rapidly than it reduces heterozygosity (Nei 1978). As such, the analysis estimates the heterozygosity expected from a sample, given the observed allelic diversity, under a model of mutation-drift equilibrium, and compares that value statistically to the heterozygosity observed in the sample. An excess of observed heterozygosity, relative to the expected, may indicate a recent bottleneck. I used the two-phase mutational model, as

recommended as most appropriate for microsatellites (Di Rienzo et al. 1994) using the sign probability test.

3.4 Results

Of the 24 microsatellite loci analyzed, none of the pairs of loci showed significant linkage disequilibrium in any population. Three loci (EMusat10, EMusat13, EMusat35) showed significant deviation from HWE in four or more populations ($P<0.01$) and showed evidence of null alleles in six populations (Table 3-2). These loci were removed from all further analyses and all subsequent results were based on the remaining 21 loci.

3.4.1 Genetic diversity

The expected heterozygosity of Mottled Duskywing populations ranged from 0.45 (Michigan and Oakville) to 0.59 (New York) (Table 3-3). The observed heterozygosity of Mottled Duskywing populations ranged from 0.39 (Oakville) to 0.53 (New York) (Table 3-3). Allelic richness of Mottled Duskywing populations ranged from 2.70 (Oakville) to 3.99 (Manitoba) (Table 3-3). All metrics of genetic diversity (H_E, H_O, A_R), remained relatively stable across generations for sites that were sampled both in 2019 and 2020.

Table 3-2. Mottled Duskywing microsatellite loci showing evidence of deviation from Hardy Weinberg Equilibrium and null alleles according to GENEPOP'007 software (Rousset 2008) and MICROCHECKER v2.2.3 (Van Oosterhout et al., 2004) respectively. Out of HWE refers to loci out of Hardy Weinberg Equilibrium and Evidence of Null refers to loci showing evidence of null alleles.

Locus	Out of HWE	Evidence of Null
EMusat10	Burlington 2020, New York 2020	Marmora 2019 & 2020, Burlington 2019 & 2020, Burlington Gas Line 2020, New York 2020
EMusat13	Burlington 2019 & 2020, Rice Lake 1 2019 & 2020, New York 2020, Manitoba 2020	Marmora 2019, Burlington 2019 & 2020, Rice Lake 1 2020, New York 2020, Manitoba 2020
EMusat35	Marmora 2019 & 2020, Burlington 2019 & 2020, Burlington Gas Line 2020, Rice Lake 1 2020, Oakville 2020, Rice Lake 3 2020, Manitoba 2020	Marmora 2019, Burlington 2019 & 2020, Burlington Gas Line 2020, Rice Lake 1 2020, New York 2020

Table 3-3. Diversity metrics calculated using R statistical software (R Core Team 2020) with packages “adegenet” (Jombart 2008), “pegas” (Paradis 2010), “hierfstat” (Goudet 2005), and “diveRsity” (Keenan et al. 2013), from microsatellite data obtained from Mottled Duskywing populations in Ontario, Manitoba, Michigan, and New York.

Site	H_E	H_O	A_R
Marmora 2019	0.54	0.51	3.18
Marmora 2020	0.52	0.50	3.26
Rice Lake 1 2019	0.50	0.50	3.59
Rice Lake 1 2020	0.54	0.49	3.52
Rice Lake 2 2020	0.47	0.46	3.20
Rice Lake 3 2020	0.53	0.50	3.07
Burlington 2019	0.56	0.48	3.26
Burlington 2020	0.56	0.49	3.62
Burlington Gas Line 2020	0.55	0.48	3.63
Oakville 2020	0.45	0.39	2.70
Manitoba 2020	0.58	0.51	3.99
Michigan 2019	0.45	0.43	3.05
Michigan 2020	0.51	0.51	3.37
New York 2020	0.59	0.53	3.90

3.4.2 Population structure

3.4.2.1 *Population differentiation*

Fixation Index (F_{ST}) for population pairs ranged from 0.0182 (Rice Lake 1 and Rice Lake 2) to 0.2381 (Marmora and Rice Lake 3) (Table 3-4). Populations showed a significant correlation between log geographic distance and F_{ST} ($r_{45,43}$=0.26, p<0.05; Figure 3-3). Low pairwise F_{ST} was observed between populations separated by less than approximately 8 km (i.e., log distance = 0.9 km), and pairwise F_{ST} values between populations separated by greater distances were much higher on average but showed no clear trend of increasing with increasing geographic distance.

Table 3-4. Pairwise F_{ST} between populations of Mottled Duskywing in Ontario, New York, Michigan, and Manitoba. F_{ST} values calculated using genotypes at 21 microsatellite loci with GENEPOP'007 software (Rousset 2008).

Site	Burlington	Burlington Gas Line	Manitoba	Marmora	Michigan	New York	Oakville	Rice Lake 1	Rice Lake 2
Burlington									
Burlington Gas Line	0.052								
Manitoba	0.119	0.081							
Marmora	0.147	0.152	0.173						
Michigan	0.116	0.134	0.144	0.155					
New York	0.110	0.115	0.106	0.155	0.101				
Oakville	0.170	0.167	0.191	0.222	0.207	0.150			
Rice Lake 1	0.132	0.126	0.126	0.191	0.166	0.080	0.168		
Rice Lake 2	0.163	0.151	0.170	0.193	0.188	0.099	0.161	0.018	
Rice Lake 3	0.176	0.174	0.198	0.238	0.237	0.134	0.142	0.069	0.044

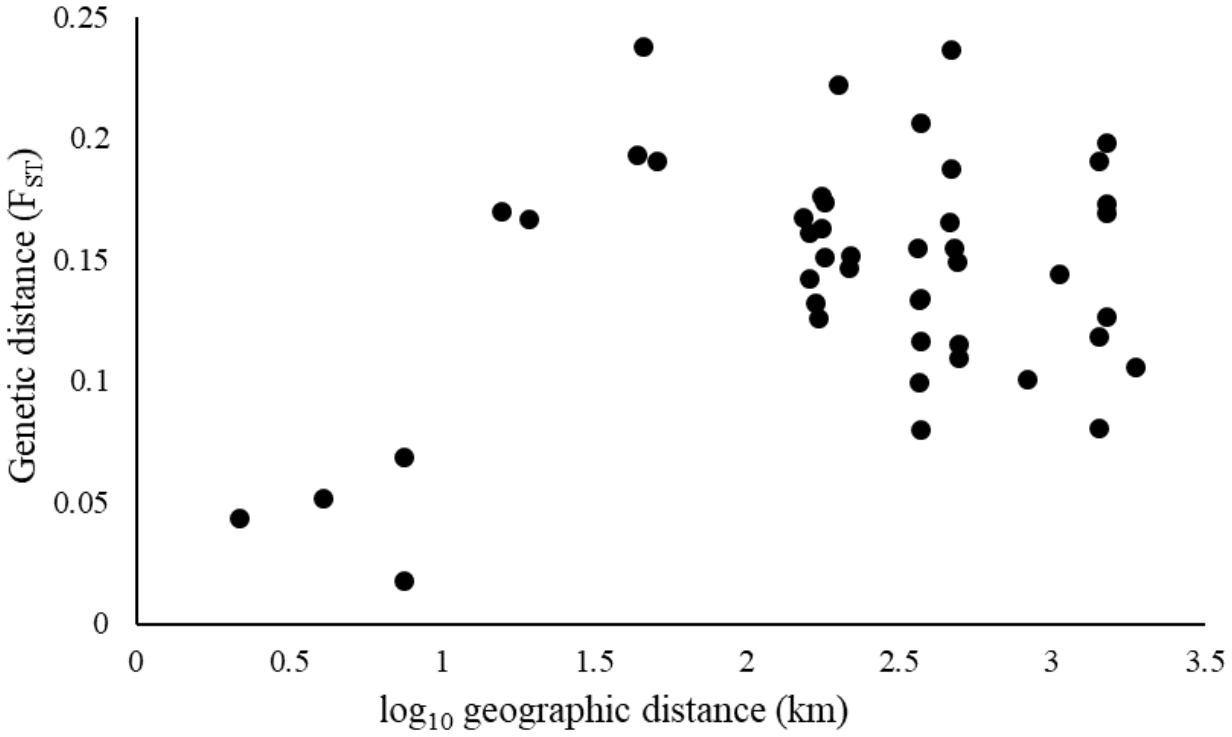

Figure 3-3. The relationship between genetic differentiation (F_{ST}), estimated using 21 microsatellites, and log transformed geographic distance (km) between pairs of populations of the Mottled Duskywing in Ontario, New York, Michigan, and Manitoba.

3.4.2.2 *Discriminant analyses of principal components*

Using DAPC, the samples were sorted into nine suggested groups (Figure 3-4). The Marmora 2019 and 2020 samples clustered together strongly (Figure 3-4, group 1). Additionally, samples from both sampling years at Burlington, along with the Burlington Gas Line 2020 sample, were clustered together into two groups (3 and 7). The Rice Lake Area sampling sites (Rice Lake 1 2019, Rice Lake 1 2020, Rice Lake 2 2020, Rice Lake 3 2020) were clustered together into two groups (5 and 9). The USA sites (New York 2020, Michigan 2019, Michigan 2020) were also clustered together in two different groups (6 and 8). Oakville 2020 was differentiated from all other groups (4) as was Manitoba 2020 (2).

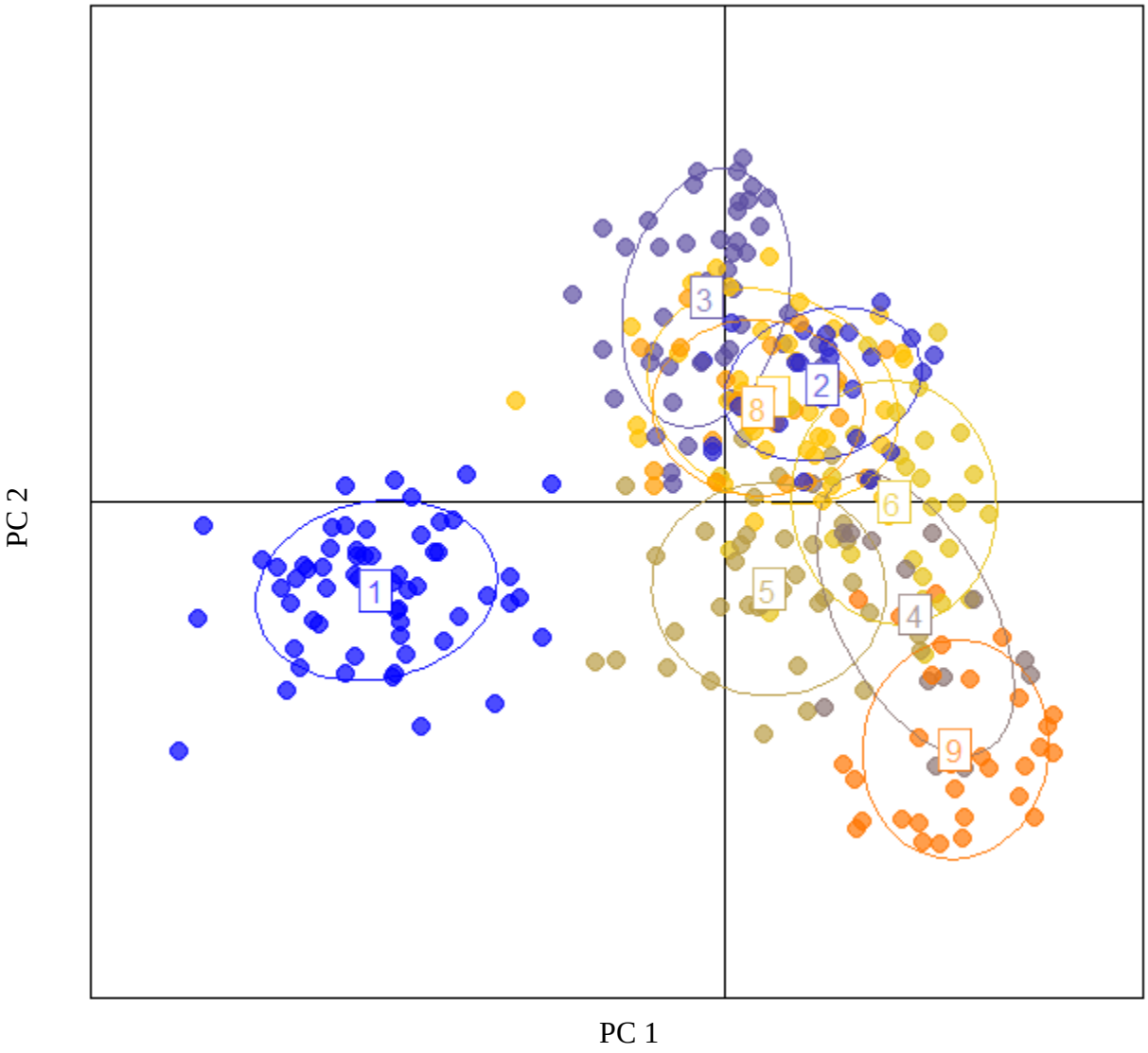

Figure 3-4. Clustering of Mottled Duskywing samples, based on microsatellite data, using Discriminant Analysis of Principal Components (DAPC). Individuals are represented as dots and identified clusters represented by colour and inclusion of 95 % inertia ellipses placed on the plane using first two principal components.

3.4.2.3 *STRUCTURE analysis*

Like the DAPC analysis, STRUCTURE also grouped the samples into nine populations, based on MaxMeanK (Puechmaille 2016) (Figure 3-5). Marmora 2019 and 2020 were grouped together (Figure 3-5; blue). Rice Lake Plains samples were grouped together, although in two populations, one composed of N 2019 and 2020 (Figure 3-5, light pink) and the other composed of Rice Lake 2 2020 and Rice Lake 3 2020 (Figure 3-5, green), with some admixture between them. Both Burlington sampling years were grouped together (Figure 3-5, orange), with subtle mixing with the Burlington Gas Line 2020 sample (Figure 3-5, deep pink). All the USA samples were grouped together (Michigan 2019 and 2020, and New York 2020; Figure 3-5, purple). Oakville 2020 (Figure 3-5, light green) and Manitoba 2020 (Figure 3-5, bright pink) formed separate populations. The ninth cluster (Figure 3-5, yellow) is represented by subtle admixture across most populations.

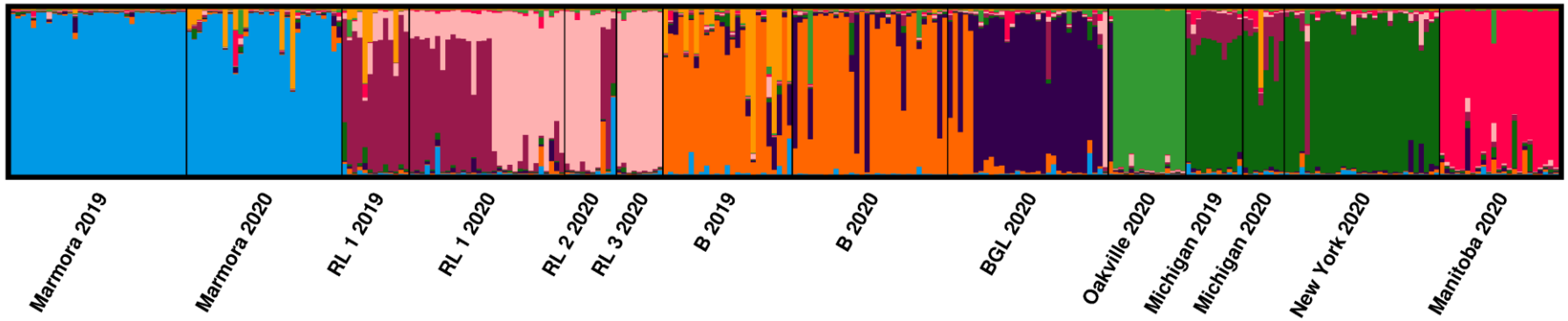

Figure 3-5. Clustering of Mottled Duskywing samples from Ontario, New York, Michigan, and Manitoba, based on 21 microsatellite loci, using STRUCTURE (Pritchard et al. 2000; Falush et al. 2003), STRUCTURE SELECTOR (Li and Liu 2018), and CLUMPAK (Kopelman et al. 2015). Each vertical line represents a sampled individual and is divided into coloured segments representing admixture assignment to nine genetic clusters. RL denotes Rice Lake; B denotes Burlington; BGL denotes Burlington Gas Line.

3.4.3 Demographic stability

3.4.3.1 *Effective population size*

Estimated N_e ranged from 5.9–963.4 (Table 3-5). Estimates of "infinite" in the confidence intervals result when there is no evidence of drift, or temporal variation in allele frequencies once sampling error is accounted for (Waples and Do 2010). The largest effective population size estimated was for Burlington (approximately 963), followed by Marmora (approximately 35). Michigan and Rice Lake 1 had similar effective population sizes to each other, but considerably lower than the other two populations (approximately 6 for both).

Table 3-5. Variance in effective population sizes (N_e) for sampled Mottled Duskywing populations, with sample sizes above five individuals in both 2019 and 2020, based on microsatellite data, estimated by NeEstimator v2 (Do et al. 2014) using the linkage disequilibrium method (Waples and Do 2008). The last column presents the 95 % confidence intervals of effective population sizes.

Site	# of samples collected in 2019/2020	Estimated N_e (# of individuals)	95 % C.I. (# of individuals
Burlington	25/30	963.4	28.2-Infinite
Michigan	11/8	6.3	3.1-17.2
Rice Lake 1	13/30	5.9	3.4-10.7
Marmora	34/30	34.8	13.8-335.5

3.4.3.2 *Bottleneck tests*

Analyses using BOTTLENECK indicated no significant evidence of a recent bottleneck occurring in any of the populations tested (all p>0.05; Table 3-6).

Table 3-6. Tests for evidence of recent bottlenecks in Mottled Duskywing populations, for populations from which > 15 individuals were sampled. Analyses were conducted using BOTTLENECK software (Cornuet and Luikart 1997) under the two-phase model (Di Rienzo et al. 1994). 'Probability' indicates p-value determined by the sign test.

Site	Probability
Marmora 2019	0.34
Marmora 2020	0.21
Rice Lake 1 2020	0.45
Burlington 2019	0.13
Burlington 2020	0.09
Burlington Gas Line 2020	0.54
Oakville 2020	0.10
New York 2020	0.59
Manitoba 2020	0.57

3.5 Discussion

3.5.1 Genetic diversity

Overall, Mottled Duskywing populations had moderate levels of genetic diversity, compared to estimates obtained using microsatellite markers in other butterflies (Table 3-7). Among all Mottled Duskywing samples analyzed here, mean overall H_E was 0.525 and mean overall H_O was 0.484. These values are similar to, or slightly lower than heterozygosity values observed in other butterfly species using microsatellite data (Table 3-3, Table 3-7). Mean overall A_R across all Mottled Duskywing samples was 3.381, which again is similar to, or slightly lower than estimates from other species (Table 3-3, Table 3-7). In general, species that are not threatened tend to have higher genetic diversity metrics compared to the threatened and endangered groups; Mottled Duskywing genetic diversity metrics are comparable with other threatened and endangered butterfly species (Table 3-3, Table 3-7). However, there are several threatened or endangered species that have fairly high genetic diversity estimates (e.g., *Lycaena helle*; Trense et al. 2021). This can occur if populations are large enough to buffer against genetic drift and inbreeding (Trense et al. 2021) or have a long history of population isolation such that they are better able to cope with small population size even if isolated (Habel and Schmitt 2012). Alternatively, high diversity rates might reflect historically high diversity that has not yet been lost due to a time-lag (Essl et al. 2015).

Table 3-7. Genetic diversity metrics reported from previous studies of butterfly species using microsatellites. H_E refers to expected heterozygosity, H_O refers to observed heterozygosity, and A_R refers to allelic richness. NR refers to metrics that were not reported in the article. I conducted a search on Google Scholar using the terms "allelic richness microsatellite butterfly" or "expected heterozygosity microsatellite butterfly" and selected 30 representative species that are categorized as either endangered, threatened or not threatened for comparison with genetic diversity levels in the Mottled Duskywing.

Species	Status	H_E	H_O	A_R	Study
Argynnis nerippe	Endangered	0.183–0.985	0.324–0.863	2.274–6.755	Jeong et al. 2018
Cyclargus thomasi bethunebakeri	Endangered	0.458–0.601	0.362–0.510	2.988–3.121	Saarinen et al. 2014
Euphydryas aurinia	Endangered	0.233–0.521	NR	2.212–1.148	Sigaard et al. 2008
Lycaena helle	Endangered	0.76–0.85	0.42–0.50	6.1–7.3	Trense et al. 2021
Melitaea protomedia	Endangered	0.396–0.662	0.434–0.600	2.22–3.47	Nakahama & Isagi 2017
Neonympha mitchellii francisci	Endangered	0.431–0.594	0.371–0.560	2.91–4.3	Milko et al. 2012
Oarisma poweshiek	Endangered	0.347–0.507	0.127–0.480	1.54–2.20	Saarinen et al. 2016
Parnassius apollo	Endangered	0.251–0.954	0.075–0.796	NR	Petenian et al 2005
Parnassius apollo filbricus	Endangered	0.46–0.60	0.53–0.59	3.05–4.05	Martínez et al. 2018
Phengaris arion	Endangered	0.611–0.742	0.500–0.705	4.33–7.51	Rutkowski et al. 2009
Zizina emelina	Endangered	0.06–1.00	0.06–0.85	NR	Sato et al. 2020
Boloria aquilonaris	Threatened	0.294–0.576	NR	0.888–1.445	Turlure et al. 2014
Danaus plexippus	Threatened	0.415–0.631	0.394–0.484	NR	Lyons et al. 2012
Erynnis propertius	Threatened	0.709–0.903	0.319–0.816	2.9–3.9	Zakharov & Hellmann 2007
Lycaena hippothoe	Threatened	0.64–0.78	0.44–0.66	5.0–7.2	Trense et al. 2021
Maculinea alcon	Threatened	0.32–0.65	NR	2.0–3.5	Vanden Broeck et al. 2017
Maculinea arion	Threatened	0.45–0.57	NR	NR	Andersen et al. 2014
Phengaris alcon	Threatened	0.46–0.61	0.42–0.67	3.10–5.89	Sielezniew et al. 2011
Phengaris nausithous	Threatened	0.70–0.71	NR	6.2–7.8	Ritter et al. 2013
Phengaris rebeli	Threatened	0.156–0.267	0.070–0.237	1.77–2.16	Rutkowski et al. 2009
Phengaris rebeli	Threatened	0.13–0.55	0.07–0.55	1.60–3.93	Sielezniew et al. 2011
Phengaris teleius	Threatened	0.41–0.67	NR	1.5–3.3	Ritter et al. 2013
Arhopala epimuta	Not threatened	0.690–0.778	0.362–0.441	7.16–8.51	Fauvelot et al. 2006
Erebia palarica	Not threatened	0.812–0.818	NR	6.7–8.39	Vila et al. 2009
Maniola jurtina	Not threatened	0.713–0.805	0.279–0.902	7.765–9.096	Greenwell et al. 2021
Papilio zelicaon	Not threatened	0.432–0.866	0.206–0.813	3.0–5.2	Zakharov & Hellmann 2007
Pararge aegeria	Not threatened	0.776–0.898	0.659–842	9.667–15.333	Vandewoestijne & Van Dyck 2010
Parnassius smintheus	Not threatened	0.72–0.78	NR	5.70–6.69	Keyghobadi et al. 2005
Polyommatus coridon	Not threatened	0.899–0.919	0.697–0.784	15.66–18.74	Habel et al. 2014
Polyommatus icarus	Not threatened	0.623–0.639	0.550–0.627	7.4–8.1	Piszter et al. 2021

There was not a large variation in the estimates of diversity among populations of Mottled Duskywing (Table 3-3), despite some sites being visibly much smaller than others and apparently containing many fewer butterflies (based on encounter and captures rates in the field). A potential explanation is that most smaller populations and patches are connected to larger ones through a metapopulation system, that allows them to maintain diversity via immigration despite being small (Aycrigg and Garton 2014). This may be the case for example in the Rice Lake area where only ten butterflies were captured at both Rice Lake 2 and Rice Lake 3, but diversity is still moderate and there is evidence of admixture with the larger population at Rice Lake 1 (Figure 3-4; Figure 3-5). Another potential explanation for moderate diversity in the smaller populations is that because habitat loss and decline have been recent, there is a time-lag in the loss of diversity, and populations have not yet reached an equilibrium level of diversity (Essl et al. 2015). Supporting this is the fact that A_R is more variable among the populations (lowest in the small, isolated population of Oakville); A_R approaches equilibrium faster than H_E and H_O, exemplified by A_R having stronger correlations with recent changes to population structure in other butterflies (Caplins et al. 2014).

3.5.2 Population structure

Though genetic differentiation and geographic distance were positively correlated, the correlation was not strong (Figure 3-3). This significant but weak IBD pattern appears to be due to low genetic differentiation of populations that are close together, within approximately 8 km specifically, and higher average differentiation of populations that are more than 8 km apart. Thus, rather than a gradual linear increase of differentiation with distance, there appears to be a threshold where populations more than

8 km apart are more highly differentiated from each other (Figure 3-3). It is also important to note that beyond the 8 km separation, populations no longer show any association between geographic and genetic distance and there is considerable variance in pairwise F_{ST} values. High mean and variability in pairwise genetic differentiation values, and a lack of association with geographic distance, are indicative of a dominant effect of genetic drift in determining genetic patterns and a lack of appreciable gene flow among populations (Hutchison & Templeton 1999). The IBD plot for the Mottled Duskywing therefore suggests a shift in the relative influence of genetic drift versus gene flow with spatial scale, such that gene flow between populations is influential at smaller distances below approximately 8-10 km, but genetic drift is dominant at larger scales (Hutchison & Templeton 1999). These IBD results were further supported in the DAPC and STRUCTURE analyses where it was primarily samples from the same locations representing different years, or sites near each other, that grouped together (Figure 3-4; Figure 3-5).

Genetic differentiation tends to reflect limited dispersal among populations (Bohonak 1999), so high differentiation, and limited or no gene flow, among populations further than 8 km apart suggest Mottled Duskywings have limited dispersal ability at that scale. Since most extant populations of Mottled Duskywing are separated by more than 8-10 km, they are likely isolated from each other. This limited dispersal ability, and wide separation of areas of suitable habitat, may limit the natural recolonization ability of *Erynnis martialis*, underscoring the importance of translocation and reintroduction for the species. On the other hand, given evidence of potential connectivity below approximately 8 km, future management plans for extant or reintroduced Mottled Duskywing

populations may want to consider managing sites within an 8 km distance a single management unit. Management plans might also consider restoring habitat within 8 km of occupied sites to promote natural colonization and establishment of new populations. Furthermore, future studies should address how the abundance and distribution of habitat, and other landscape features, may affect movement and gene flow among populations at this scale.

An interesting result was the relatively low F_{ST} value (0.101) between the New York and Michigan populations, and their grouping in both DAPC and STRUCTURE analyses, despite an approximate 831 km distance (Figure 3-4; Figure 3-5). Contemporary dispersal between these two locations is very unlikely given their spatial separation. One possible explanation for their genetic similarity is that it is a legacy of historical connectivity, when populations and suitable habitats were much more widespread. However, high genetic differentiation among currently extant populations that are much closer together, such as Burlington and Marmora in Ontario, do not lend support to this hypothesis. The most parsimonious explanation for the apparent genetic similarity of these distant populations is simply that it is a consequence of the dominant effect of random genetic drift, which introduces high variation in pairwise population differentiation, independent of geographic distance (Hutchison and Templeton 1999). Homoplasy (identity in state but not by descent), which can occur due at microsatellites because of their high mutation rates and allele size constraints (Putman and Carborne 2014), may also be a contributing factor.

3.5.3 Demographic stability

Effective population sizes, for the four populations where I had sufficient sampling to estimate this parameter, were moderate to low, except for Burlington where the upper confidence interval indicated no evidence of drift after accounting for sampling error. A study of *Hipparchia semele*, a butterfly species of conservation concern that showed evidence of inbreeding, estimated an N_e range of 20–54 individuals using similar methods (De Ro et al. 2021). Effective population size estimates for the threatened *Maculinea alcon* butterfly using microsatellites were much lower at 1.6–17.6 individuals (Vanden Broeck et al. 2017). As the program required a substantial sample size for both sampling years, Mottled Duskywing N_e was only estimated for large sites where more individuals were sampled, so these results may be biased due to ease of sampling at sites with highest density of Mottled Duskywing. Overall, my results indicate that all but the largest populations of the Mottled Duskywing are likely subject to appreciable levels of genetic drift.

While I did not find significant evidence of recent bottlenecks in any populations, a review on genetic bottleneck testing suggests that these tests often fail to detect declines in populations, even when they are known to have occurred (Peery et al. 2012). The low power of bottleneck tests may be due to factors such as short duration of the bottleneck, immigration, and high pre-bottleneck genetic diversity, all of which can dampen or quickly eliminate the expected genetic signature (Peery et al. 2012). Although I cannot definitively rule out the occurrence of bottlenecks, particularly in populations for which sample sizes were too small for testing, my results do not point to recent bottlenecks as a basis for selection, or exclusion, of any potential source populations for reintroduction.

3.5.4 Recommendations for reintroduction

The reintroduction of the Mottled Duskywing to Pinery Provincial Park in Grand Bend, Ontario, has begun. The two main populations originally considered as a source were Marmora and Burlington. As the reintroduction was to occur in Ontario, an Ontario source population was preferred. Among known populations in the province, Marmora and Burlington seemed to have the highest abundance of Mottled Duskywing, based on the number of butterflies captured or observed during the flight season. Additionally, they were logistically good choices for reintroduction because they were easy to access, and permissions from landowners were readily obtained. From my study, and preliminary results that I had collected before the summer 2021 reintroduction to Pinery Provincial Park, I knew that both potential source populations held similar levels of genetic diversity. However, I also knew that they were highly differentiated from one another (based on pairwise F_{ST} and STRUCTURE analyses). Therefore, evaluating the genetic data I had obtained within the context of the adaptative potential strategy, I had recommended that either population would be a suitable source. For comparison, the previously mentioned successful reintroduction of *Maculinea arion* reported a mean estimated H_E of the reintroduced populations between 0.45–0.57, which was not significantly different from the H_E of the source population (Andersen et al. 2014). Estimated H_E for both Marmora (H_E = 0.52 in 2019 and H_E = 0.54 in 2020) and Burlington (H_E = 0.56 in both years) are within that range. Furthermore, given that each population had moderate levels of genetic diversity, but they were highly differentiated from each other, population mixing was not necessary or recommended. The recommendation to avoid mixing source populations was also supported by preliminary

testing I conducted showing evidence of infection by the reproductive parasite *Wolbachia* in both the Marmora and Burlington populations (Shayla Kroeze, unpublished data). In general, mixing of populations in reintroductions of the Mottled Duskywing should be avoided until further testing to determine the prevalence of *Wolbachia*, and the identity of strains in different populations, has been conducted (Dincă et al 2018).

It would be useful to continue attempts to extract DNA from historical specimens, where those are available, to determine the genetic make-up of extirpated populations that previously occupied proposed reintroduction sites. This information could inform founder selection based on ancestry match (Houde et al. 2015). In terms of environment match, an entire group of associated butterflies in the UK showed evidence of temperature-mediated local adaptation related to emergence timing (Roy et al. 2015). Mottled Duskywing have been observed to have one or two broods per year depending on geographic range (Layberry et al. 1998). Future reintroductions or population augmentation of the Mottled Duskywing in Ontario, such as the planned reintroduction to Norfolk County, should investigate environmental factors such as those that impact emergence timing that may influence genetic variation in Mottled Duskywings and may affect how well-adapted founder individuals are to local conditions in a reintroduction site. Important environmental characteristics may be subtle, as in the example of *Hesperia comma*, where the size of the host plant and the state of the surrounding ground cover were found to be important for the suitability of oviposition (Thomas et al. 1986). Detailed studies of habitat and environmental requirements may be particularly important for Mottled Duskywing considering populations have become extirpated from habitat that appears to be suitable, with abundant host plant and nectaring plants (personal obs.).

3.6 Conclusion

I used 24 microsatellite markers previously described in Chapter 2 to characterize genetic diversity, population structure, and demographic stability of Mottled Duskywing populations in Ontario and nearby locations. Overall, populations had moderate levels of genetic diversity. I also found evidence for high average genetic differentiation, and limited gene flow, among populations separated by more than approximately 8 km. I used this genetic information to inform the selection of the Marmora population as a suitable source population for reintroduction to Pinery Provincial Park.

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Chapter 4

4 General Discussion

I successfully developed microsatellite markers for the endangered Mottled Duskywing butterfly and characterized genetic diversity and structure of populations in Ontario, New York, Michigan, and Manitoba. My work represents the first research on Mottled Duskywing population genetics. Overall, populations had moderate levels of genetic diversity and, in most instances, were highly genetically differentiated from each other. This information was used to inform source population selection for the first reintroduction of this species, as part of its recovery strategy; in the summer flight season of 2021, over 700 adult butterflies, larvae, and pupae reared in captivity from females collected in the Marmora population were released to Pinery Provincial Park (Groleau 2021). The field crew at Pinery confirmed that reintroduced butterflies mated and laid eggs there (Ontario BSAR 2021). In addition to informing this reintroduction and future planned reintroductions, my research has filled key knowledge gaps about Mottled Duskywing biology and genetics. My results suggest that alternative source populations such as the Burlington or Rice Lake 1 populations could be used for future reintroductions to different locations in Ontario to prevent reducing genetic resources or causing excessive disturbance to the Marmora population, as they have comparable levels of genetic diversity.

4.1 Future applications and research

The genetic tools that I developed can now be used to monitor the genetic status of introduced and current populations. All reintroduced populations should be monitored according to IUCN guidelines, including genetic monitoring (IUCN/SSC 2013). Information from ongoing monitoring can inform the optimum number and size of further releases and identify unexpected threats to reintroduced populations. Estimation of key demographic and genetic parameters of a reintroduced population is critical to define reintroduction success and inform adaptive management strategies (DeMay et al. 2017).

The microsatellites I developed may also prove useful for selecting individual butterflies for captive rearing programs to ensure maximum genetic diversity. For example, adult females that provide eggs for the captive rearing program could be genotyped prior to any releases of their offspring. If any of these females are found to be very genetically similar, their offspring could be more effectively partitioned among different areas of a reintroduction site to distribute the genetic diversity and reduce inbreeding. Alternatively, a large number of potential egg-laying females could be collected in the wild and genotyped using non-lethal tissue samples before eggs are laid, and then only the most genetically diverse group retained for captive breeding. Given that captive rearing is very labour intensive, this approach could preserve time and resources used for captive breeding while maximizing genetic diversity of the reared offspring group.

In this thesis I made recommendations for source population selection based primarily on the adaptive potential strategy and ensuring sufficient genetic diversity among founders but balanced against considerations of outbreeding and potential effects of reproductive parasites. There is still scope to investigate alternate strategies for source population selection, specifically pre-existing adaptation strategies including environment matching and ancestry matching (Houde et al. 2015), for future reintroductions of the Mottled Duskywing. Future studies may investigate important environmental conditions for Mottled Duskywing survival, which would be necessary for developing any environment matching strategy. Building on such work, potentially functional or expressed genetic markers (e.g., SNPs) could be developed to allow research strategies such as genome–environment association (GEA) analyses that link functional genes to individual environmental predictors (Pluess et al. 2016). Considering Mottled Duskywing populations had moderate levels of genetic diversity, identification of large numbers of SNPs may be possible from even a small number of individuals sampled from a single population, using methods such as genotyping by sequencing (GBS; Deschamps et al. 2012) or restriction associated DNA sequencing (RADSeq: Andrews et al. 2016). These data could then be used to develop assays for moderate numbers (e.g., hundreds) of SNPs that could be genotyped using the small amounts of DNA obtained from non-lethal tissue samples. Though no GEA analyses have been conducted for butterfly species, there is evidence that fire regimes increased genetic diversity in two butterfly species (Gates et al. 2021). This study may be especially relevant to the Mottled Duskywing that is known to have a relationship with fire as the host plant will decline due to succession in the absence of disturbance (COSEWIC 2015; COSEWIC 2012).

Research that continues to attempt to extract DNA from, and genotype, historical specimens should also be further explored. Information about historical allele frequencies of Mottled Duskywings, especially in populations that have become extirpated from sites that are potential targets of reintroduction, would be necessary for ancestry matching. A combination of strategies could also be employed where if multiple potential source populations exhibit evidence for pre-existing adaption, either through environment or ancestry match, the population that also has a higher level of genetic diversity would be selected. Also, research to determine *Wolbachia* prevalence, and characterize strains of *Wolbachia*, in different populations is important for a better understanding of the risks associated with mixing individuals from different populations, either for sourcing reintroductions or augmenting declining populations.

4.2 Conservation implications

Considering that Mottled Duskywing populations show moderate levels of genetic diversity, conservation efforts directed towards extant populations might focus on threats other than low genetic diversity, such as habitat degradation, climate change, and pesticide use (COSEWIC 2015). Augmentation of current populations may not be necessary, although this view may be biased considering analysis was often not possible at sites where very few individuals were sampled, likely due to low butterfly abundance. The Oakville population exhibited the lowest amount of genetic diversity of the Ontario populations based on all three metrics (H_E, H_O, A_R). However, there are existing plans to construct a road within critical habitat for that population. If possible, nearby habitat should be restored, and the population could be augmented with butterflies from other

populations so the population can persist. However, considering the Oakville population has high genetic differentiation from all other sites and we still do not have information about *Wolbachia* variation in Mottled Duskywing populations, augmentation is only recommended if the population is on the brink of extinction.

Most populations I studied were genetically isolated from each other, meaning that each population represents a unique subset of the total genetic diversity of the Mottled Duskywing. This highlights the importance of conserving all existing populations to conserve overall genetic diversity. Additionally, persistence of different subsets of diversity across populations increase the chances of finding potential future matches (populations that have similar allele frequencies and/or exist in similar environments) if the pre-existing adaptation strategy were to be employed for future reintroductions (Houde et al. 2015).

Several studies have looked at population genetics of butterfly species post-reintroduction (Davis et al. 2021; Kuussaari et al. 2015; Andersen et al. 2014; Schmitt et al. 2005; Irmgard 2001). Fewer studies have examined butterfly population genetics to inform source population selection prior to reintroduction, one making recommendations based on ancestry matching (Saarinen and Daniels 2012), and one based on selecting the closest population geographically, or environment matching (Gunson 2019), two different approaches within the pre-existing adaptation strategy. Future results from the reintroduction of the Mottled Duskywing will provide critical information on the success of the adaptive potential strategy for selection of a source population. I hope that my work can serve as a model for similar conservation and reintroduction projects on other

species, especially insect species that make up so much of the Earth's diversity (Stork 2018; Prather et al. 2013).

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5 Appendices

Appendix A: Number of observed alleles per microsatellite locus per Mottled Duskywing population in Ontario, New York, Michigan, and Manitoba

Locus	Population	# of alleles
EMusat2	Burlington 2019	5
	Burlington 2020	5
	Burlington Gas Line 2020	6
	Manitoba 2020	6
	Marmora 2019	4
	Marmora 2020	3
	Michigan 2019	2
	Michigan 2020	5
	New York 2020	6
	Oakville 2020	5
	Rice Lake 1 2019	6
	Rice Lake 1 2020	5
	Rice Lake 2 2020	4
	Rice Lake 3 2020	5
EMusat3	Burlington 2019	2
	Burlington 2020	2
	Burlington Gas Line 2020	4
	Manitoba 2020	4
	Marmora 2019	5
	Marmora 2020	5
	Michigan 2019	5
	Michigan 2020	4
	New York 2020	4
	Oakville 2020	3
	Rice Lake 1 2019	3
	Rice Lake 1 2020	4
	Rice Lake 2 2020	3
	Rice Lake 3 2020	4

EMusat4	Burlington 2019	7
	Burlington 2020	7
	Burlington Gas Line 2020	8
	Manitoba 2020	11
	Marmora 2019	9
	Marmora 2020	8
	Michigan 2019	5
	Michigan 2020	7
	New York 2020	10
	Oakville 2020	3
	Rice Lake 1 2019	6
	Rice Lake 1 2020	7
	Rice Lake 2 2020	5
	Rice Lake 3 2020	0
EMusat5	Burlington 2019	2
	Burlington 2020	2
	Burlington Gas Line 2020	3
	Manitoba 2020	2
	Marmora 2019	2
	Marmora 2020	2
	Michigan 2019	1
	Michigan 2020	1
	New York 2020	3
	Oakville 2020	2
	Rice Lake 1 2019	1
	Rice Lake 1 2020	2
	Rice Lake 2 2020	1
	Rice Lake 3 2020	2
EMusat8	Burlington 2019	12
	Burlington 2020	13
	Burlington Gas Line 2020	14
	Manitoba 2020	11
	Marmora 2019	7
	Marmora 2020	7
	Michigan 2019	11
	Michigan 2020	11
	New York 2020	16
	Oakville 2020	6
	Rice Lake 1 2019	10
	Rice Lake 1 2020	12
	Rice Lake 2 2020	9
	Rice Lake 3 2020	6

EMusat9	Burlington 2019	3
	Burlington 2020	5
	Burlington Gas Line 2020	4
	Manitoba 2020	3
	Marmora 2019	3
	Marmora 2020	4
	Michigan 2019	2
	Michigan 2020	3
	New York 2020	3
	Oakville 2020	3
	Rice Lake 1 2019	4
	Rice Lake 1 2020	3
	Rice Lake 2 2020	3
	Rice Lake 3 2020	4
EMusat10	Burlington 2019	4
	Burlington 2020	5
	Burlington Gas Line 2020	5
	Manitoba 2020	6
	Marmora 2019	5
	Marmora 2020	6
	Michigan 2019	4
	Michigan 2020	5
	New York 2020	5
	Oakville 2020	4
	Rice Lake 1 2019	5
	Rice Lake 1 2020	5
	Rice Lake 2 2020	5
	Rice Lake 3 2020	5
EMusat11	Burlington 2019	3
	Burlington 2020	2
	Burlington Gas Line 2020	4
	Manitoba 2020	2
	Marmora 2019	2
	Marmora 2020	2
	Michigan 2019	1
	Michigan 2020	3
	New York 2020	2
	Oakville 2020	2
	Rice Lake 1 2019	3
	Rice Lake 1 2020	4
	Rice Lake 2 2020	2
	Rice Lake 3 2020	2

EMusat12	Burlington 2019	3
	Burlington 2020	2
	Burlington Gas Line 2020	3
	Manitoba 2020	4
	Marmora 2019	2
	Marmora 2020	2
	Michigan 2019	2
	Michigan 2020	2
	New York 2020	6
	Oakville 2020	1
	Rice Lake 1 2019	3
	Rice Lake 1 2020	2
	Rice Lake 2 2020	1
	Rice Lake 3 2020	1
EMusat13	Burlington 2019	2
	Burlington 2020	3
	Burlington Gas Line 2020	3
	Manitoba 2020	4
	Marmora 2019	3
	Marmora 2020	2
	Michigan 2019	2
	Michigan 2020	3
	New York 2020	2
	Oakville 2020	3
	Rice Lake 1 2019	3
	Rice Lake 1 2020	3
	Rice Lake 2 2020	2
	Rice Lake 3 2020	3
EMusat14	Burlington 2019	3
	Burlington 2020	2
	Burlington Gas Line 2020	4
	Manitoba 2020	3
	Marmora 2019	4
	Marmora 2020	4
	Michigan 2019	2
	Michigan 2020	3
	New York 2020	4
	Oakville 2020	4
	Rice Lake 1 2019	2
	Rice Lake 1 2020	3
	Rice Lake 2 2020	3
	Rice Lake 3 2020	3

EMusat16	Burlington 2019	3
	Burlington 2020	4
	Burlington Gas Line 2020	5
	Manitoba 2020	4
	Marmora 2019	3
	Marmora 2020	4
	Michigan 2019	3
	Michigan 2020	2
	New York 2020	5
	Oakville 2020	2
	Rice Lake 1 2019	4
	Rice Lake 1 2020	4
	Rice Lake 2 2020	4
	Rice Lake 3 2020	3
EMusat17	Burlington 2019	3
	Burlington 2020	3
	Burlington Gas Line 2020	3
	Manitoba 2020	3
	Marmora 2019	2
	Marmora 2020	3
	Michigan 2019	3
	Michigan 2020	2
	New York 2020	2
	Oakville 2020	4
	Rice Lake 1 2019	2
	Rice Lake 1 2020	2
	Rice Lake 2 2020	1
	Rice Lake 3 2020	2
EMusat18	Burlington 2019	7
	Burlington 2020	7
	Burlington Gas Line 2020	6
	Manitoba 2020	9
	Marmora 2019	6
	Marmora 2020	4
	Michigan 2019	3
	Michigan 2020	4
	New York 2020	7
	Oakville 2020	4
	Rice Lake 1 2019	6
	Rice Lake 1 2020	6
	Rice Lake 2 2020	6
	Rice Lake 3 2020	4

EMusat20	Burlington 2019	4
	Burlington 2020	4
	Burlington Gas Line 2020	3
	Manitoba 2020	4
	Marmora 2019	5
	Marmora 2020	2
	Michigan 2019	3
	Michigan 2020	3
	New York 2020	4
	Oakville 2020	2
	Rice Lake 1 2019	2
	Rice Lake 1 2020	2
	Rice Lake 2 2020	2
	Rice Lake 3 2020	2
EMusat22	Burlington 2019	3
	Burlington 2020	3
	Burlington Gas Line 2020	3
	Manitoba 2020	5
	Marmora 2019	4
	Marmora 2020	4
	Michigan 2019	3
	Michigan 2020	2
	New York 2020	3
	Oakville 2020	5
	Rice Lake 1 2019	4
	Rice Lake 1 2020	3
	Rice Lake 2 2020	2
	Rice Lake 3 2020	6
EMusat25	Burlington 2019	2
	Burlington 2020	4
	Burlington Gas Line 2020	2
	Manitoba 2020	3
	Marmora 2019	2
	Marmora 2020	2
	Michigan 2019	3
	Michigan 2020	2
	New York 2020	4
	Oakville 2020	5
	Rice Lake 1 2019	3
	Rice Lake 1 2020	3
	Rice Lake 2 2020	2
	Rice Lake 3 2020	3

EMusat27	Burlington 2019	3
	Burlington 2020	3
	Burlington Gas Line 2020	3
	Manitoba 2020	3
	Marmora 2019	2
	Marmora 2020	2
	Michigan 2019	2
	Michigan 2020	2
	New York 2020	2
	Oakville 2020	1
	Rice Lake 1 2019	2
	Rice Lake 1 2020	2
	Rice Lake 2 2020	2
	Rice Lake 3 2020	2
EMusat31	Burlington 2019	6
	Burlington 2020	7
	Burlington Gas Line 2020	5
	Manitoba 2020	9
	Marmora 2019	6
	Marmora 2020	7
	Michigan 2019	5
	Michigan 2020	5
	New York 2020	9
	Oakville 2020	2
	Rice Lake 1 2019	6
	Rice Lake 1 2020	6
	Rice Lake 2 2020	5
	Rice Lake 3 2020	3
EMusat34	Burlington 2019	8
	Burlington 2020	10
	Burlington Gas Line 2020	8
	Manitoba 2020	10
	Marmora 2019	5
	Marmora 2020	6
	Michigan 2019	5
	Michigan 2020	5
	New York 2020	9
	Oakville 2020	5
	Rice Lake 1 2019	7
	Rice Lake 1 2020	7
	Rice Lake 2 2020	6
	Rice Lake 3 2020	6

EMusat35	Burlington 2019	3
	Burlington 2020	5
	Burlington Gas Line 2020	4
	Manitoba 2020	4
	Marmora 2019	5
	Marmora 2020	6
	Michigan 2019	2
	Michigan 2020	3
	New York 2020	6
	Oakville 2020	5
	Rice Lake 1 2019	3
	Rice Lake 1 2020	4
	Rice Lake 2 2020	3
	Rice Lake 3 2020	3
EMusat36	Burlington 2019	9
	Burlington 2020	8
	Burlington Gas Line 2020	8
	Manitoba 2020	7
	Marmora 2019	5
	Marmora 2020	7
	Michigan 2019	6
	Michigan 2020	5
	New York 2020	8
	Oakville 2020	6
	Rice Lake 1 2019	8
	Rice Lake 1 2020	9
	Rice Lake 2 2020	8
	Rice Lake 3 2020	6
EMusat37	Burlington 2019	6
	Burlington 2020	6
	Burlington Gas Line 2020	5
	Manitoba 2020	8
	Marmora 2019	5
	Marmora 2020	6
	Michigan 2019	6
	Michigan 2020	6
	New York 2020	6
	Oakville 2020	4
	Rice Lake 1 2019	5
	Rice Lake 1 2020	6
	Rice Lake 2 2020	6
	Rice Lake 3 2020	5

EMusat40	Burlington 2019	7
	Burlington 2020	8
	Burlington Gas Line 2020	10
	Manitoba 2020	10
	Marmora 2019	6
	Marmora 2020	7
	Michigan 2019	8
	Michigan 2020	6
	New York 2020	10
	Oakville 2020	9
	Rice Lake 1 2019	7
	Rice Lake 1 2020	10
	Rice Lake 2 2020	7
	Rice Lake 3 2020	6

Curriculum Vitae

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Publications:

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