The population and conservation genetics of the Marsh Fritillary butterfly *Euphydryas aurinia* in the British Isles.

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This thesis is submitted to Edge Hill University in partial fulfilment of the degree of Doctor of Philosophy

May 2019

Contents

Abstract

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This thesis investigates the ecological genetics of the Marsh Fritillary butterfly in the British Isles using microsatellite markers. This is broken down into four questions. The first question to be addressed is what is the population differentiation across a broad landscape? The second question asks what the population differentiation is on a similar geographical scale when populations are isolated by water? The third question asks what management units can be identified at local and regional scales? The final question addresses the genetic composition and diversity of a reintroduced population with reference to the source populations.

This study suggests that movements of <400m occur frequently and within that distance populations cannot be considered separate even when occupying discreet patches of habitat. Movements of >4km occur but infrequently enough for patches this distance apart to be considered separate populations, with significant pairwise Fst values detected at this range. Previous studies, mainly based on observation and recapture studies, have estimated the dispersal range to be between 300m-20km. The upper limit of dispersal was not determined but the 20km previous proposed is commensurate with this study.

Strong evidence for spacial structuring and population differentiation was found over relatively short distances (<12km). Isolation by distance was observed only in the most geographically separated dataset (Ireland), suggesting that at shorter distances (<100km) the landscape matrix may have more of an effect on dispersal than straight line distance. Water is not a barrier to dispersal and pairwise Fst for island populations is similar to equivalent mainland population pairs over the same distance. It is also theorised that multi-generational stepping-stone dispersal may occur in both terrestrial and mixed terrestrial-open water habitats.

Levels of genetic diversity in reintroduced populations whose donor stock was an admixture bred from two separate areas was found to be similar or higher than that of natural populations. Populations reintroduced from the same captive stock began to show population differentiation nine generations after reintroduction.

Most populations examined exhibited low observed heterozygosity and departures from Hardy-Weinberg equilibrium, implying that this is normal for the species. Genetic variation is unevenly distributed across the landscape although analysis showed that this was not easily explained by landscape features. The management implications of findings are discussed and suggest genetic evidence can inform management decisions. Specifically, genetic diversity is not equally distributed across the landscape and this needs to be taken into consideration if planning landscape level intervention. When designating management units, sites which are <400m apart should be treated as a single population and managed together, while sites which are >4km apart are separate populations and should be treated as separate management units.

Keywords: *Euphydryas aurinia*; Marsh Fritillary; Lepidoptera; ecological genetics; conservation; gene flow; dispersal; reintroduction.

Acknowledgments

Although it is my name on the thesis, there are numerous people without whom this work would not have been possible.

I am grateful to everyone who has assisted with sample collection over the course of the project. For Scotland my especial thanks to David Wood and Andy Knight of the RSPB and Gordon Campbell of SNH for their assistance and sample collection. For Cumbria my thanks to Steve Doyle and the members of the Cumbrian Marsh Fritillary Action Group. For Cornwall, my thanks to Robin Curtis of University of Exeter for sample collecting with me even through the snow storms. For Ireland, my thanks to Rebecca Whitla for sharing samples collected for her MRes project (and through her my thanks to those who assisted with her sample collection), also to Jesmond Harding and his volunteers at Butterfly Conservation Ireland for the material they sent. For Wales I am indebted to Jon Hudson of the University of Birmingham for the dataset and Niall McKeown from Aberystwyth University for the initial analysis. I am also grateful to Marc Botham at CEH although we were never able to sample at Salisbury Plain. For historical specimens I am grateful to the curators of Oxford University Museum, Manchester Museum, Liverpool World Museum, Tullie House Museum and the Natural History Museum for access to their specimens. I am also grateful to Tim Melling and Peter Summers for allowing sampling of their private collections.

My thanks are also owed to all landowners and site managers for allowing access to their lands for sampling and to Natural England, Scottish Natural Heritage, Countryside Council Wales/Natural Resources Wales and the Northern Ireland Department of Agriculture, Environment and Rural Affairs for granting licences for this work to be carried out. My gratitude also to my field assistants Rebecca Whitla, Matt

Esh, Josh Styles and notably Mark Ashton for his willingness to drive round Scotland for weeks and lack of hesitation in climbing over fences to get just one more sample.

Within the Department of Biology, I would like to thank Emily Joseph, Vicky Gentile, Claire Lane and Louise Jenkinson (Admins past and present) without whom I would have been lost in paperwork. I am also grateful to all the Technicians, especially Dave Goodson for putting up with all my lab requests. For technical assistance with lab and analysis methods (especially R code) I am grateful to my fellow PhD students Thom Dallimore, Carl Barker and Elizabeth Sullivan.

I am beyond grateful to my supervisors Paul Ashton and Ian Powell for giving me this opportunity and for reading countless drafts full of my 'idiosyncratic' spellings and interesting use of the English language, and for correcting the same. I am thankful to all members of the department, past and present, for the interesting and bizarre lunchtime chats and for being there when I just wanted someone to talk to. Especial thanks go to Thom, Tom and Becca for the Tech Hub chats which went everywhere but frits and mozzies.

On a personal level I need to thank all my friends for their support throughout this degree, with special thanks to my best friend Ashley Bhalla who kept me something resembling sane even at the worst times. Thanks must also go to my family who have supported me through not just this degree but also all the time before that and encouraged me to pursue my dreams. With special thanks to my mum Ann Richardson and my uncle Lyndon Davis without whom I don't think I would have ended up where I am.

1 Introduction

1.1 The Biodiversity Crisis

The world is currently experiencing a biodiversity crisis on a scale comparable to the mass extinctions (Wake & Vredenburg, 2008; Barnosky *et al*., 2011). The present rate of extinction is at least a hundred times higher than the background rate (Ceballos *et al*., 2015; De Vos *et al*., 2015) and this elevation in the extinction rate is driven, directly and indirectly, by human activity (Ceballos *et al*., 2015).

On 6th May 2019 the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services (IPBES) released a Global Assessment summary (Diaz *et al*., 2019). It found that 75% of land surface area and 66% of ocean area were showing significant impacts and alterations. It also found that the average abundance of native species in the major biomes had dropped by 20% and that 25% of assessed plant and animal species were threatened with extinction, amounting to over 1 million species at risk of extinction, some within decades. It also concluded that climate change was the greatest direct driver of environmental change due to how it exacerbated the impact of other drivers such as land use change. Land use change, notable the conversion of forest for agriculture, had the largest independent negative impact on the environment, this impact is then exacerbated by the effects of climate change leading to a greater negative impact over all.

This unprecedented rate of change and loss has led to a desire to conserve remaining biodiversity. This desire can be motivated by philosophical, moral or religious reasons, generally summarised as a desire to leave the natural world to the next generation in the same, or better, condition than we received it (Paterson, 2006; van Houtan, 2006; Negi, 2010). It can also be motivated by more pragmatic reasons. For example preserving the functioning of the ecosystem services on which humanity

depends, such as food crop pollination and the provision of clean air and water (De Groot *et al*., 2002; Pagiola *et al*., 2004; Spangenberg & Settele, 2010).

In considering the conservation of biodiversity, there are three levels of biodiversity recognised by both the Convention on Biological Diversity and the IUCN: Ecosystem, species, and genetic (UNEP, 1992; Meynell *et al*., 2010). However, practical conservation work and projects tend to focus upon the ecosystem and species levels, for the clear reason that these are visible and therefore easier to attract attention and funding.

1.2 The Conservation of Genetic Diversity

Conservation of genetic diversity is the least addressed of the three components of biodiversity. However genetic diversity is of critical importance as it provides the variation upon which natural selection can act. In the short term a lack of genetic diversity causes inbreeding depression and other associated problems which can lead to population declines and increased extinction risk. In the long term it limits the ability of a species to adapt to changes in the environment such as climate or a novel pathogen (Allendoff *et al.,* 2013).

Genetic diversity has two components; richness (the amount of diversity) and evenness (how it is apportioned) (Lowe *et al*., 2004). Genetic richness is the measure of allelic diversity in a population or group of populations. This can be measured in various ways including allelic richness, mean number of polymorphic loci and observed heterozygosity. In contrast genetic evenness is a measure of the differentiation between populations, how evenly the genetic diversity is spread across all populations. This is commonly measured using diversity indices such as Nei's Gst (Nei, 1973) (the coefficient of genetic differentiation) and Wright's F-statistics (Wright, 1951) (fixation indicies describing the level of heterozygosity in a population). It is

important to understand both aspects of genetic diversity within a species when planning and carrying out both *in-situ* and *ex-situ* conservation.

Understanding the natural apportionment of genetic diversity *in-situ* can help to detect populations with relatively low genetic diversity and thus which are at risk of inbreeding depression. Assessments of genetic diversity can also be used to measure gene flow between populations. This information can then be used to guide other conservation activities such as the creation of habitat corridors or stepping stones, or artificial translocations should it be necessary. Such a translocation was deemed necessary in the case of the Florida panther (*Felis concolor coryi*), a subspecies of mountain lion. The Florida panther persisted for many generations as a small remnant population (~40 breeding adults); it exhibited low genetic variation compared to other mountain lion subspecies and physiological characteristics indicative of inbreeding (Hedrick, 1995). Eight Texas Puma females (*F. c. stanleyana*) were released into Florida in 1995 where they successfully bred. Following the release, what is termed a genetic rescue, population numbers dramatically increased, as did measures of fitness and survival (Johnson *et al*., 2010). The species also expanded into habitat that had previously been considered incapable of supporting it and it is clear that the genetic rescue increased the probability of the sub-species persisting the wild, though as a hybrid of *F. c. coryi* and *F. c. stanleyana* (Pimm *et al*., 2006).

For *ex-situ* conservation consideration of genetic diversity is important at a number of stages. This includes, when possible, the selection of founder stock for minimum kinship and maximum diversity and then the continued management of the captive stock to minimise kinship and any deleterious recessive disorders. This occurred with the captive breeding of the California condor (*Gymnogyps californianus*). The breeding programme was founded with the remaining 14 individuals in 1987 and in 1998 five severely deformed embryos were produced which displayed chondrodystrophy, an autosomal recessive disorder which results in a form

of dwarfism which is lethal before or immediately after hatching. Based on the high frequency of the allele (9%) at the time it was discovered, it was determined that more than half of the captive breeding population were potential carriers (78 out of 146). From this it was estimated that three of the founders of the captive breeding program were carriers for the allele (Ralls *et al*, 2000). The population was managed to reduce the expression of the lethal phenotype, by separation of affected pairs, and to generally minimise kinship within the population (Ralls & Ballou, 2006).

1.3 The Global Lepidoptera Declines

The declines of charismatic megafauna have generated the greatest public interest and support, as evidenced by the case of the Giant Panda which was recently reclassified from Endangered to Vulnerable (Swaisgood *et* al., 2016). Declines in invertebrate species have recieved significantly less attention despite the vital role of invertebrates in ecological functions (Prather *et al*., 2013). However the sparse data available suggests invertebrate declines are just as severe as those seen in vertebrate species (Dunn, 2005; Régnier *et al*., 2015). Approximately 1% of the estimated 1.4 million invertebrate species have been assessed by the IUCN Red List and of these 40% are listed as threatened, however those assessed do not represent a random selection.

Lepidoptera have probably received more interest than other invertebrate orders. However, this interest has not prevented declines and extinctions and it is clear that butterflies are declining globally (Sánchez-Bayo & Wyckhuys, 2019). Since they are sensitive to changes in their environment and are often used as an indicator species, it may reasonably be assumed that if butterflies are declining then other specialist invertebrate species are as well (New, 1997a; Kerr *et al*., 2000; Gossner *et al.*, 2014).

Species loss is greatest in the tropics, but it is also seen in temperate regions. In the UK declines in either extent of occurrence, abundance or both have been reported in 76% of resident or regular migrant butterfly species in the past 40 years (Fox *et al*., 2015). Moths have not been monitored as extensively but of the 337 common and widespread species, 227 have declined over the same 40-year period, with half of those that have declined having done so by more than 50% (Fox *et al*., 2013).

Conservation activities which aim to halt or reverse this decline tend to be species specific in temperate regions rather than the broader habitat protection approaches used in the tropics (Bonebrake *et al.*, 2010). This focus on species specific work includes understanding the population genetics associated with Lepidoptera conservation. At present the literature regarding conservation genetics in Lepidoptera is primarily limited to descriptive studies documenting the population genetics of a single species at a specific site and suggesting that it may have conservation implications (For example: Meglécz *et al.*, 1997; Cassel & Tammaru, 2003; Joyce & Pullin, 2003; Vandewoestijne & Baguette, 2004; Vila *et al.*, 2006; Meng *et al*., 2008; Junker & Schmitt, 2010; Pecsenye *et al.*, 2018). Other studies document the effect of isolation or fragmentation on genetic diversity in a particular species but do not offer broadly applicable management recommendations (For example: Keyghobadi *et al.*, 1999a; Joyce, 2001; Harper *et al.*, 2003; Keyghobadi *et al.*, 2005; Orsini *et al*., 2008; Sigaard *et al.*, 2008; Smee, 2011; Martínez *et al.*, 2017). This lack of broadly applicable rules means that the population genetics of each species of conservation concern must be investigated individually.

1.4 The Marsh Fritillary

The Marsh Fritillary butterfly (Figure 1.1) appears in Lepidoptera guide books from at least 1895 (Kappel *et al*., 1895), with some museum specimens and recorded observations dating from the early half of the nineteenth century (Natural History Museum, 2014). A member of the Nymphalidae family, the scientific name has been subject to some revisions over time with the species being reclassified into several different genera (*Melitaea*, *Papilio* and *Eurodryas*). The presently accepted scientific name is *Euphydryas aurinia* (Rott. 1775).

Figure 1.1 Adult Marsh Fritillary butterfly at rest, Glenborrodale (Scotland) June 2014. Photograph by Kirsty Godsman.

The Marsh Fritillary is broadly distributed throughout the northern temperate regions in Eurasia, from Ireland across to Korea and from approximately 35°N to 62°N and is declining throughout its range (van Swaay *et al*., 2010). However the

species/subspecies status of the populations in China and the surrounding region are subject to debate (Korb *et al*., 2016). Within Europe there have, at various times, been more than fifty subspecies of the Marsh Fritillary recognised (Beccaloni *et al*., 2019) As these are still subject to debate it is sometimes referred to as the *E. aurinia* species complex (Munguira *et al*., 1997; Casacci *et al*., 2015).

The most recent taxonomic revision of the *E. aurinia* complex across its full range was by Korb *et al.* (2016) which used a combination of molecular techniques (COI barcoding) and morphological characteristics (male and female genital structures and wing pattern). They identified six *Euphydryas* species (*E. aurinia, E. beckeri, E. discordia, E. sibirica, E. laeta* and *E. asiatica*) and 14 subspecies (six within *E. aurinia,* three within *E. sibirica* and five within *E. laeta*) based primarily on morphological analysis with some supported by additional molecular evidence. Although the study did not include any of the taxa of the British Isles, the geographical ranges of the various *E. aurinia* subspecies described by Korb *et al*. would suggest that *E. aurinia* ssp *aurinia* is the native subspecies. However, there has been no modern taxonomic treatment of the purported subspecies within the British Isles.

The problem of subspecies is exacerbated by the confusion over taxonomic rank, which has included the ranks of subspecies, forms and races. Descriptions are based on differences in wing colouration; primarily colour contrast and melanism patterns exhibited. One of the earliest treatments was by Birchall (1873) who described *E. aurinia* f. *hibernica* as representative of the Irish populations. In contrast Kane (1893) described *E. aurinia* f. *praeclara* as the common Irish form along with *E. aurinia.* f. *signifera* in Penarth, Wales, while Robson (1889) added *E. aurinia* f. *scotica* as representative of the Scottish populations. Fruhstorfer (1916) identified two subspecies *E. aurinia* ssp*. anglicana* in England (described specimen was from Kent) and *E. aurinia* ssp. *acedia* in Wales (and south-west England).

Ford (1945) however identified only two subspecies: *E. a. aurinia* which was found in England, Scotland and Wales, with a few populations in Ireland, and *E. a.* ssp. *praeclara* being widespread in Ireland and Oban in Scotland (*praeclara* had previously been considered a form). Subsequent taxonomic revisions raised *hibernica* to subspecies rank (Kloet, 1972) and classified ssp*. praeclara* as a junior synonym of ssp*. hibernica* (Dennis, 1977). This is the most recent formal taxonomic treatment of the species in the British Isles however the Marsh Fritillary continues to appear in handbooks. Some handbooks ignore sub-specific taxa completely (e.g. Asher *et al*., 2001; Fox *et al.*, 2015). However, Thomas & Lewington (1991) identify forma *hibernica* in contrast to Kloet 1972 who classified it as a subspecies rather than a form*.* There is no reference to subspecies in UK or EU legislation.

There is some evidence that Britain was colonised in a single slow colonization event (Joyce & Pullin, 2001) and that from this Ireland was subsequently also colonised in a single event (Whitla, 2019). Despite the possibly limited numbers involved in the colonisation of the various parts of the British Isles, Ford (1945) commented that “this is an exceptionally variable species” and that variation is on a continuum. Given the lack of molecular taxonomic treatments and Ford’s view on the variation, the question of subspecies will not be further addressed in this thesis, and all populations considered only as *E. aurinia*, the Marsh Fritillary.

Within the British Isles the Marsh Fritillary is found in two habitat types: Damp, neutral or acidic pastures and dry calcareous grasslands. (Barnett & Warren, 1995). It is suggested that damp pastures are its native habitat and that the spread onto dry grasslands has occurred within the last century following changes in grazing regimes (Warren, 1994). The larval host plant in both habitats is Devil’s-bit scabious, *Succisa pratensis* (Moench) (Figure 1.2), although larvae will feed on other plants such as field scabious (*Knautea arvensis*) and small scabious (*Scabiosa columbaria*), especially in later instars (Porter, 1981).

Figure 1.2. Devil's-bit Scabious in flower on Mull, August 2019. Photograph by Ian Powell.

Larvae hatch in late-July/early-August (depending on latitude and microclimate, Figure 1.3), they are gregarious, living in silken webs and feed through the first three instar stages until September (Figure 1.4). The fourth stage instar diapauses over winter and emerges in February/March to bask (Figure 1.5). Fifth instar are less gregarious and begin to feed independently, the sixth instar is solitary and will pupate in April/May. Adults emerge and are in flight from late-May through to mid-July. Female Marsh Fritillaries mate soon after emergence and will lay an initial batch of up to 500 eggs in the natal patch. Additional smaller batches may be laid in other patches (Porter, 1981, 1982).

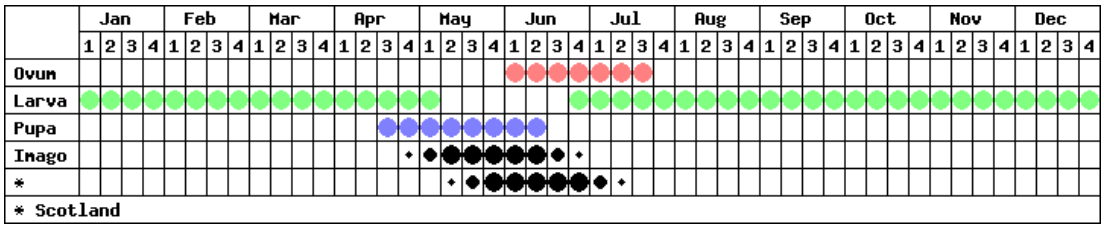

Figure 1.3 Life cycle of the Marsh Fritillary butterfly indicating the times of year at which each life stage can be found. Note that all stages are affected by latitude, with larvae in Scotland usually entering diapause later than those in south England. From UK Butterflies (2018).

Figure 1.4. Third instar larvae feeding on Devil's-bit scabious leave within and without the silken web they spin for protection. Note from the first to third instar larvae are brown in colour. Cumbria, August 2016.

Figure 1.5. Fourth instar larvae, recently emerged from diapause, basking following snow. Note that from the fourth instar onwards larvae are black in colour. Cornwall, March 2018.

The Marsh Fritillary has a classical/Levins metapopulation dynamic (Warren, 1994). Within this dynamic individual populations are subject to large fluctuations in size, including patch extinction and recolonistation (Hanski & Gaggiotti, 2004). In the Marsh Fritillary such dramatic fluctuations in individual populations have been observed since the earliest studies (Ford & Ford, 1930). Some of these fluctuations are the impact of weather patterns (Butterfly Conservation, 2019), however parasitoid attack is also known to play a major role in population fluctuations (Botham *et al.*, 2011).

The Marsh Fritillary is subject to attack by the two larval parasitoid wasps *Cotesia bignelli* and *C. melitaearum*; these parasitoids can have up to three generations per host generation, allowing it to decimate populations (Porter, 1981; Bulman, 2001; Klapwijk & Lewis, 2014). Although *C. melitaearum* will parasitise multiple fritillary species (Kankare *et al*., 2006), including two found in the British Isles (the Glanville fritillary, *Melitaea cinxia,* and Heath fritillary, *M. athalia*), it is believed that in the British Isles, both *C. melitaearum* and *C bignelli* are host-specific to the Marsh Fritillary (Bulman, 2001). While *C. bignelli* may be broadly distributed in an area not all populations of the Marsh Fritillary will be equally affected and in some years some may be entirely free of the parasitoid wasp (Bulman, 2001).

Although the Marsh Fritillary is presently classified as Least Concern by the IUCN owing to its broad distribution (van Swaay *et al*., 2010), it is still acknowledged to be declining in both abundance and extent of occurrence and has been subject to regional extinctions, including complete loss from the Netherlands (van Swaay *et al*., 2010). The species is subject to international legal protection under Annexe II of the EU Habitats and Species Directive and the 1979 Bern Convention. It is also protected under national law in some countries such as the UK where it has, since 1998, been fully protected under the Wildlife and Countryside Act 1981 and is a Biodiversity Action

Plan (BAP) species. Despite this the Marsh Fritillary has declined in Britain by 73% over a 25 year period (Fox *et al*., 2010). There has been evidence in recent years that the decline may be slowing, Fox *et al*. (2010) reported only a 9% decline in English colonies in the preceding ten years compared to the 66% loss between 1990-2000 reported by Hobson *et al*., (2001).

Nevertheless the Marsh Fritillary continues to decline (Fox *et al*., 2015). There are presently several Marsh Fritillary conservation projects in Britain including landscape scale projects in Dartmoor, Dorset and Scotland (Ellis *et al.*, 2012; Butterfly Conservation, 2017a) and post-reintroduction population monitoring in Cumbria. The Cumbrian Marsh Fritillary Action Group (CMFAG) carried out a successful captive breeding and reintroduction program (Porter & Ellis, 2011). Reintroductions were carried out over several sites and years with the final captive stock being released in 2016. CMFAG subsequently has monitored sites and surrounding areas for signs of natural colonisations of which there have been several (Porter, pers. comm). Interestingly the Cumbrian reintroduction is the only population free of the parasitoid attack. The native parasitoid in Cumbria was *C. melitaearum*, it is considered to have become extinct in 2007 when the final Marsh Fritillary larvae in Cumbria were taken into captivity and confirmed to be free of the parasitoid (CMFAG, pers. comm). Plans exist to reintroduce either *C. melitaearum* or *C. bignelli* to the reintroduced Cumbrian Marsh Fritillary population (Porter & Ellis, 2011) but this has not yet occurred (Porter, pers. comm.).

Previous work on the conservation biology of the Marsh Fritillary has tended to focus on understanding the ecology and requirements of the species. This has included habitat requirements and use (Fowles & Smith, 2006; Smee *et al*., 2011; Brunbjerg *et al*., 2017; Pschera & Warren, 2018), movement and dispersal (Wahlberg *et al*., 2002; Junker & Schmitt, 2010; Zimmermann, Fric, *et al*., 2011; Casacci *et al*., 2015), demography and metapopulation structure (Warren, 1994; Betzholtz *et al.*,

2007; Zimmermann *et al*., 2011; Jugovic *et al*., 2018), and interactions with host-plants and parasitoids (Bulman, 2001; Klapwijk & Lewis, 2014; Meister *et al*., 2015). There have been studies modelling population persistence (Schtickzelle *et al*., 2005; Bulman *et al*., 2007) and documenting specific management practices and conservation attempts (Anthes *et al*., 2003; Hula *et al*., 2004; Porter & Ellis, 2011).

The Marsh Fritillary was one of the first subjects to be studied by the pioneering ecological geneticist E. B. Ford (1945). Surprisingly given the wider interest in the species, it has had little subsequent genetic study. Wang *et al*., (2003) investigated local population differentiation in China, concluding that there was significant differentiation consistent with a classic metapopulation. Sigaard *et al*. (2008) investigated genetic diversity of a remnant fragment in Denmark, concluding that habitat fragmentation had resulted in significant genetic drift and possible inbreeding. Joyce & Pullin (2003) investigated the population genetics of the British population and considered that there were two management units which should be conserved independently, Scotland-North England and Wales-South England. Smee (2011) investigated the population differentiation in south-west England concluding, in contrast to Joyce & Pullin (2003), that there was a significant level of population structuring across the region.

The limited number of previous studies have left gaps in our understanding of the conservation genetics of the Marsh Fritillary and thus in our ability to effectively conserve and manage the species. There is no information on the natural levels of genetic diversity and differentiation in the Marsh Fritillary where it has not been subject to significant range loss and fragmentation. Thus, there is no frame of reference by which the results of other studies can be contextualised. Also lacking is information on gene flow patterns across a landscape and at smaller geographic scales with dispersal distances varying within the literature (Table 1.1). Finally, there is no

Table 1.1 Dispersal distances for the Marsh Fritillary from the currently published literature.

Maximum dispersal distance	Author	Study method	Region and habitat	Notes
100m (one instance of 349m)	Junker & Schmitt (2010)	MMR	Algarve, Portugal. Hilly region, mixture of shrubland, fallow and intensively used meadows (total area 3.6ha)	
2km	Fric *et al* (2010)	MRR	Near Karlovy Vary, Czech Republic. Improved and unimproved humid grassland/pastures (total area 28.1ha)	Dispersal varied by sex (males dispersed further than females)
3km	Konvicka *et al* (2012)	MMR (multi year)	Near Karlovy Vary, Czech Republic. Improved and unimproved humid grassland/pastures (total area 28.1ha)	Dispersal varied by year and sex (males dispersed further than females in all years)
10-14km	Sigaard *et al* (2008)	Genetic (microsatellite)	Northern part of Jutland Peninsula, Denmark.	Gene flow detected between sites 10km and 14km apart
10km	Zimmerman *et al* (2011)	MRR	Western Bohemia, Czech Republic. 30 humid meadow patches (total area 28ha)	
15-20km	Warren (1994)	Long term data set analysis	Britain. Various	
40km	Barnett & Warren (1995)	Anecdotal	Britain	New colony found 40km from previously known colonies at well monitored location, authors note that this may be a clandestine release rather than a natural colonisation.

information currently available on the fate of genetic diversity in reintroduced populations. It is the intention of this study to address these gaps in knowledge.

1.5 Aims

This thesis aims to investigate the ecological genetics of Marsh Fritillary in the British Isles by investigating the following:

- Genetic diversity and apportionment at various scales in a large area that may represent the historical natural condition of the species. This was undertaken using the Irish populations (Chapter 1).
- Genetic variation and gene flow in island populations. This was undertaken in the Southern Inner Hebrides (Chapter 2).
- To use information on patterns of genetic variation to identify management units for conservation. This was done at two scales: Local scale using few populations situated a maximum of 4km apart, this was done in Cornwall, and larger scale covering an area ~750km^2, this was be done in South Wales (Chapter 3).
- The genetic composition of reintroduced admixture populations of the Marsh Fritillary in comparison to its two founder populations. This was undertaken with the reintroduced Cumbria population which was an admixture of original Cumbrian and western Scotland populations (Chapter 4).

1.6 Microsatellites

The work in this thesis was done using microsatellites. These are short sections of repeating DNA (also called Single Sequence or Short Section Repeats, SSRs) which

are commonly used in population genetics studies (Selkoe & Toonen, 2006). Once appropriate primers have been developed, microsatellites are relatively cheap and provide high levels of variation (Madesis *et al*., 2013). They occur frequently in the genomes of most species and mutate rapidly resulting in a high number of alleles, although this can lead to issues with homoplasy (Lowe *et al*., 2004). They are also bi-parentally inherited and, provided they are not closely linked with a gene under selection, selectively neutral. These characteristics prevent possible sex-biased dispersal patterns and local adaptation from influencing conclusions.

Data from microsatellites has been widely used to inform conservation programs for many years (Paerkau & Strobeck, 1994; Chase *et al.*, 1996; Wayne, 1996; Hedrick, 2001). The landscape scale source-sink metapopulation dynamics of the mountain lions (*Puma concolor*) of the Great Basin in the USA were determined via microsatellite analysis (Andreasen *et al.*, 2012). This provided important guidance to those managing the species as the hunting of it is permitted and over-exploitation of the source populations would have a far greater impact on the long-term persistence of the species than the over-hunting of a sink population.

Understanding population structuring is important in conservation and microsatellites are often used for this purpose. In two Australian species, the speckled dace (*Rhinichthys osculus*) and the grassland earless dragon (*Tympanocryptis pinguicolla*), microsatellite data was used to detect cryptic population structuring judged worthy of special conservation attention. At least one evolutionarily significant unit (ESU) was detected within the range of the speckled dace (Hoekzema & Sidlauskas, 2014) and three ESUs were detected in the grassland earless dragon (Carlson *et al*., 2016), importantly in the latter case each ESU would have separate legal status and protection under Australian law.

Microsatellites have been used to document the 12-17% reduction in heterozygosity between historic and modern populations of lions (*Panthera leo*) in

Africa, including the presence of so-called ghost alleles (alleles present only in the historic population) and loss of genetic diversity in what is considered a modern strong-hold for the species (Dures *et al.*, 2019). This study emphasised the importance of maintaining the genetic diversity so that the future resilience and adaptability of the species is not further compromised.

Microsatellites were selected for use in this study as they are widely used in similar population and conservation genetics studies due to their high variability, biparental inheritance and co-dominance (Lowe *et al.*, 2004; Allendorf, 2017). Although they are initially difficult and costly to develop, several microsatellites have already been developed for the Marsh Fritillary by previous authors reducing the preparation time needed for this study (Petenian *et al*., 2005; Sinama *et al*., 2011; Smee *et al*., 2013). Microsatellites have previously been used in other studies on the population genetics of the Marsh Fritillary (Sigaard *et al*., 2008; Smee, 2011) and other Lepidoptera species (Harper *et al*., 2003, 2006; Scott *et al*., 2006; Meng *et al*., 2008; Orsini *et al*., 2008; Habel *et al*., 2009; Bogdanowicz *et al*., 2015; Martínez *et al*., 2017; Nakahama & Isagi, 2018). The previous development and high levels of variation make microsatellites a suitable genetic marker for use in this study.

2 How it used to be in the old days? Genetic variation of the Marsh Fritillary in Ireland.

2.1 Introduction

When attempting to reverse the decline of a species and undertake active conservation it is important to understand the ecology of the species prior to its decline (Drew, 2005; Littlewood *et al.*, 2012; Courchamp *et al.*, 2015). This baseline knowledge may include distribution, habitat requirements, vagility and the level and apportionment of genetic variation (Courchamp *et al.*, 2015).

Reconstruction of the historic geographical range is possible through old records, though this may be subject to recording bias (Turvey *et al*., 2015; Yang *et al*., 2016). However historical records cannot provide information on the genetic diversity and gene flow patterns throughout the historic range. Understanding these parameters is critical given that a reduction in species range often leads to a reduction in population size. This in turn can lead to a reduction in genetic diversity (Frankham, 1996), though the exact details of the effect of range reduction on genetic variation are little studied (Arenas *et al*., 2012).

The natural level of genetic diversity shows marked interspecific variation; what may be regarded as normal for some species may be disconcertingly low and merit intervention in another (Leffler *et al*., 2012). In a practical scenario this may lead to conservation resources being ineffectively used; either diverting those resources away from a species which has what appears to be 'normal' levels of genetic diversity but are in fact a tiny fraction of historic levels or applying them to a species in which low levels of genetic diversity are an intrinsic component of its life history.

Studies of historic levels of genetic diversity would be the ideal to provide this baseline comparison for modern populations. However such studies are rare (Groombridge *et al*., 2012), often due to the difficulty in obtaining samples and working

with ancient DNA, which is typically highly degraded (Hykin *et al*., 2015; Nicholls, 2005; Burrell *et al*., 2015; Hykin *et al*., 2015; Weiß *et al*., 2016). In the absence of such historical measures of genetic diversity the alternative solution is to compare the levels of genetic diversity in populations of conservation concern with those in stable populations, ideally of the same species or, where this is not available, a very closely related one (Frankham *et al*., 2017).

The Marsh Fritillary butterfly, *Euphydryas aurinia* (Rott. 1775) (Lepidoptera: Nymphalidae), is broadly distributed throughout much of Europe and into Asia (Wang *et al*., 2004), though the classification of some populations as either separate species or subspecies is subject to debate and revision (Korb *et al*., 2016). It is suffering declines throughout its range, most countries report reductions of 5-30% with some countries experiencing greater losses or even complete extinctions, as in the Netherlands (van Swaay *et al*., 2010). The decline and attendant rarity of the Marsh Fritillary has led to studies into its; phylogeography (Joyce & Pullin, 2001), population genetics (Joyce & Pullin, 2003; Sigaard *et al*., 2008; Smee, 2011) and metapopulation dynamics (Wahlberg *et al.*, 2002; Hula *et al*., 2004; Schtickzelle *et al*., 2005; Bulman *et al*., 2007; Brunbjerg *et al*., 2017). However, studies of the level and apportionment of genetic variation in an area where the species has maintained its historic range have not been undertaken.

The British Isles is an archipelago of islands off the north west coast of continental Europe consisting of two principal land masses, Britain (England, Scotland and Wales) and Ireland (Northern Ireland and the Republic of Ireland), plus many smaller islands, including the Isle of Man and the Isle of Wight. The British Isles are a stronghold for the Marsh Fritillary with each country (the United Kingdom and the Republic of Ireland) accounting for 5-15% of the total European population (Van Swaay & Warren, 1999). However, the two principal islands have shown contrasting patterns of population persistence over the last 50 years.

The reduction in the range of the Marsh Fritillary in Britain began in the early 1900s, with the more severe declines occurring in the latter half of the twentieth century (Figure 2.1).

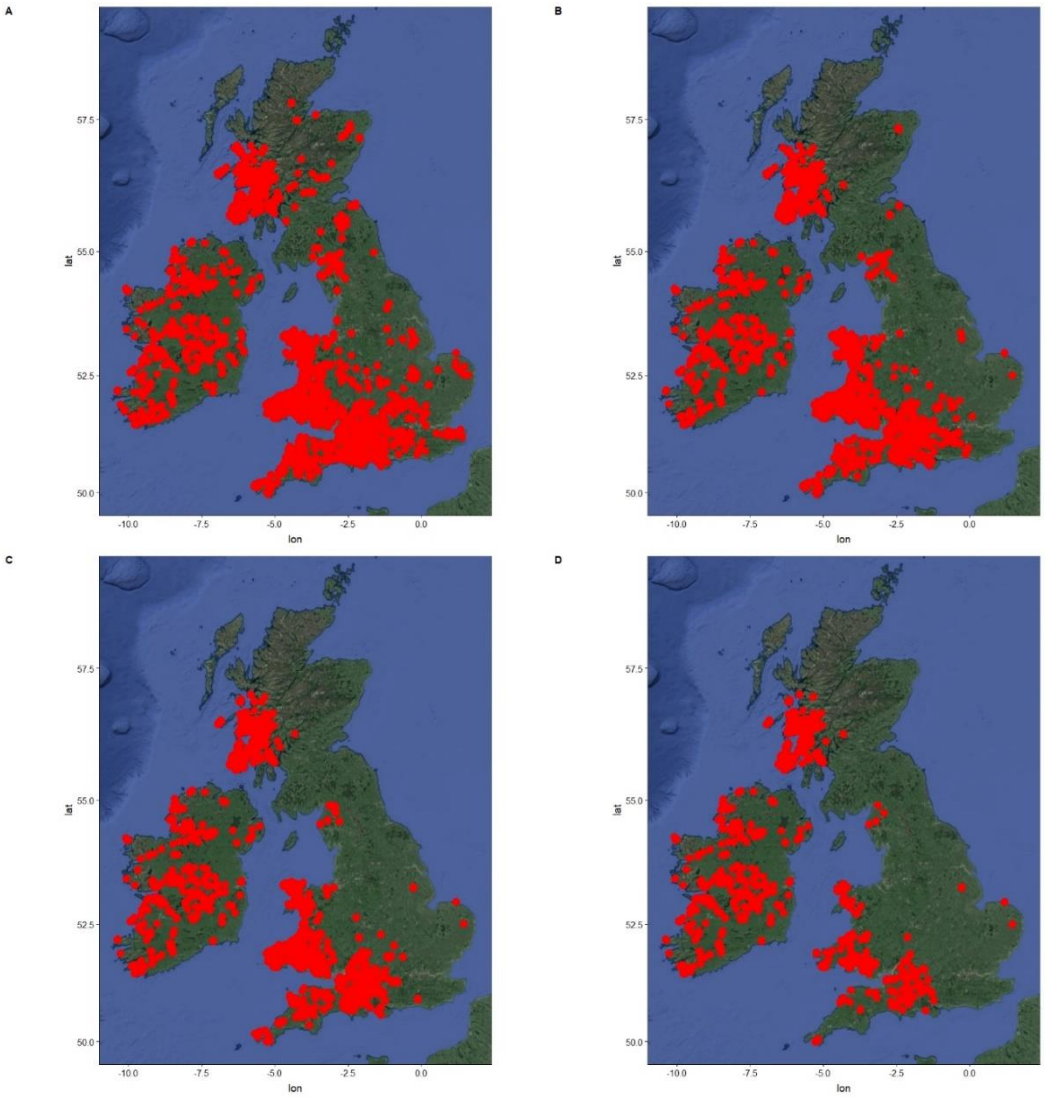

Figure 2.1. Declines of the Marsh Fritillary in the British Isles during the twentieth century. A) Pre-1900, B) 1950, C) 1975, D) 2000. Points represent records of occurrence; one point may represent multiple records at the same geographical location. Combined datasets from NBN Atlas (2019) & GBIF (2019).

This decline was in part driven by the agricultural intensification following the Second World War; prior to this, cheaper prices had caused Britain to import much of its grain and cereal crops, resulting in a reduction in tilled land compared with the late nineteenth century (Robinson & Sutherland, 2002). With the push for land reform

begun by the Scott report of 1942 (Stamp, 1943), British agricultural policy sought to increase the area under cultivation and stabilise the industry, beginning with the Agriculture Act (1947) and continuing with the Common Agriculture Policy, first established in 1962 (Robinson & Sutherland, 2002).

This policy shift combined with intensification of agriculture has resulted in a change in farming practice. At the turn of the previous century most farms were mixed, housing both crops and stock. With the pre-WWII cheap grain imports most farms had shifted towards stock and fodder crops only. The second half of the twentieth century saw a polarisation in farming practices within Britain (Figure 2.2) with the arable east and pastoral west (Robinson & Sutherland, 2002). It is notable that the post-2000 distribution of the Marsh Fritillary (Figure 2.1 D) is closely correlated with regions which have less than 40% of the land under annual tillage. Pastoral land has also been affected by the modernisation of farming practices. Improvement of grassland has been common, resulting in the replacement of species rich and structurally diverse swards with dense uniform swards dominated by a few species (Vickery *et al*., 2001). This change has negatively impacted the Marsh Fritillary by reducing the availability of its host plant Devil's-bit Scabious (*Succisa pratensis*) which is found on nutrient-poor soils (Bakker *et al*., 2002).

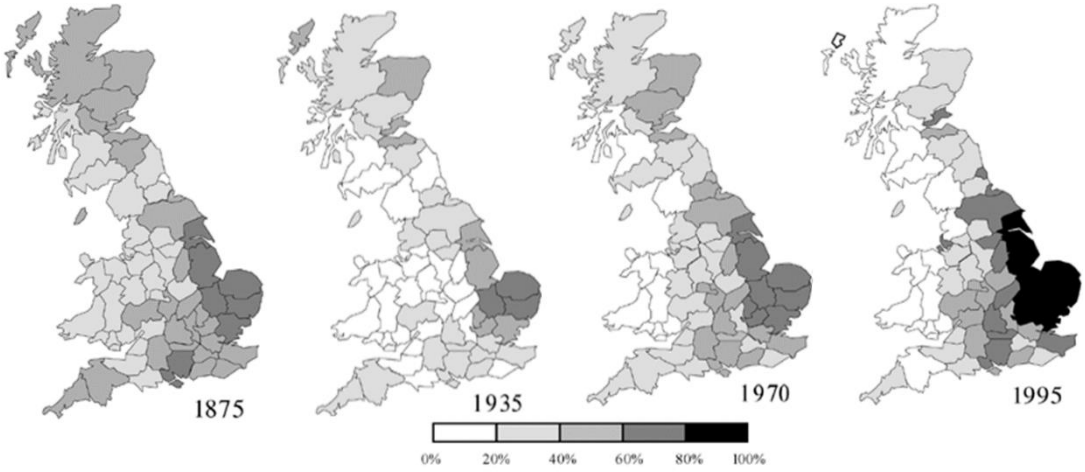

Figure 2.2. Modified from Robinson & Sutherland (2002). The percentage of land annually tilled by county based on DEFRA statistics.

The rapid decline of the Marsh Fritillary seen in Britain in the late-20th and early-21st century was, at least in part, the payment of the extinction debt owed from the rapid land use changes. Extinction debt, the future extinction of a species caused by past environmental changes (Tilman *et al*., 1994), is more commonly observed in longer lived species but is still reported in short lived ones (Bommarco *et al*., 2014), although shorter life span species respond more quickly to habitat changes so the debt may not persist for as long (Saar *et al*., 2012). Extinction debt can also be seen in metapopulation species. In this case extinction debt occurs when sufficient patches are destroyed that the dynamic shifts to a non-equilibrium metapopulation, where recolonization is no longer possible (Hanski, 1997). At this point the species is "bound for regional extinction" (Hastings & Harrison, 2003). In this scenario the species may persist in the landscape for some time, until the natural boom-bust stochasticity or further habitat changes complete the extinction (Hanski, 1997). This is what may have happened in Britain. In the mid-twentieth century the loss of some patches of habitat may have shifted the local metapopulations into non-equilibrium with the remaining individual populations persisting for several decades before going extinct in the late twentieth and early twenty-first century, accounting for the declines seen despite conservation efforts (Porter & Ellis, 2011).

In contrast, during the latter half of the twentieth century Ireland has seen both a reduction in arable land and pasture grassland due to abandonment, with natural succession resulting in a six-fold increase in forest cover from 1930 to 2000 (Eaton *et al*., 2008). However Ireland is still characterised mostly as a rural mosaic and pastoral landscape with few intensive agricultural regions (Zucaro *et al*., 2013). Present land use maps show a clear contrast between Ireland and Britain (Figure 2.3). Large areas of Britain are predominantly arable land interspersed with pastures while in Ireland the reverse is true, being predominantly pasture with small areas of arable.

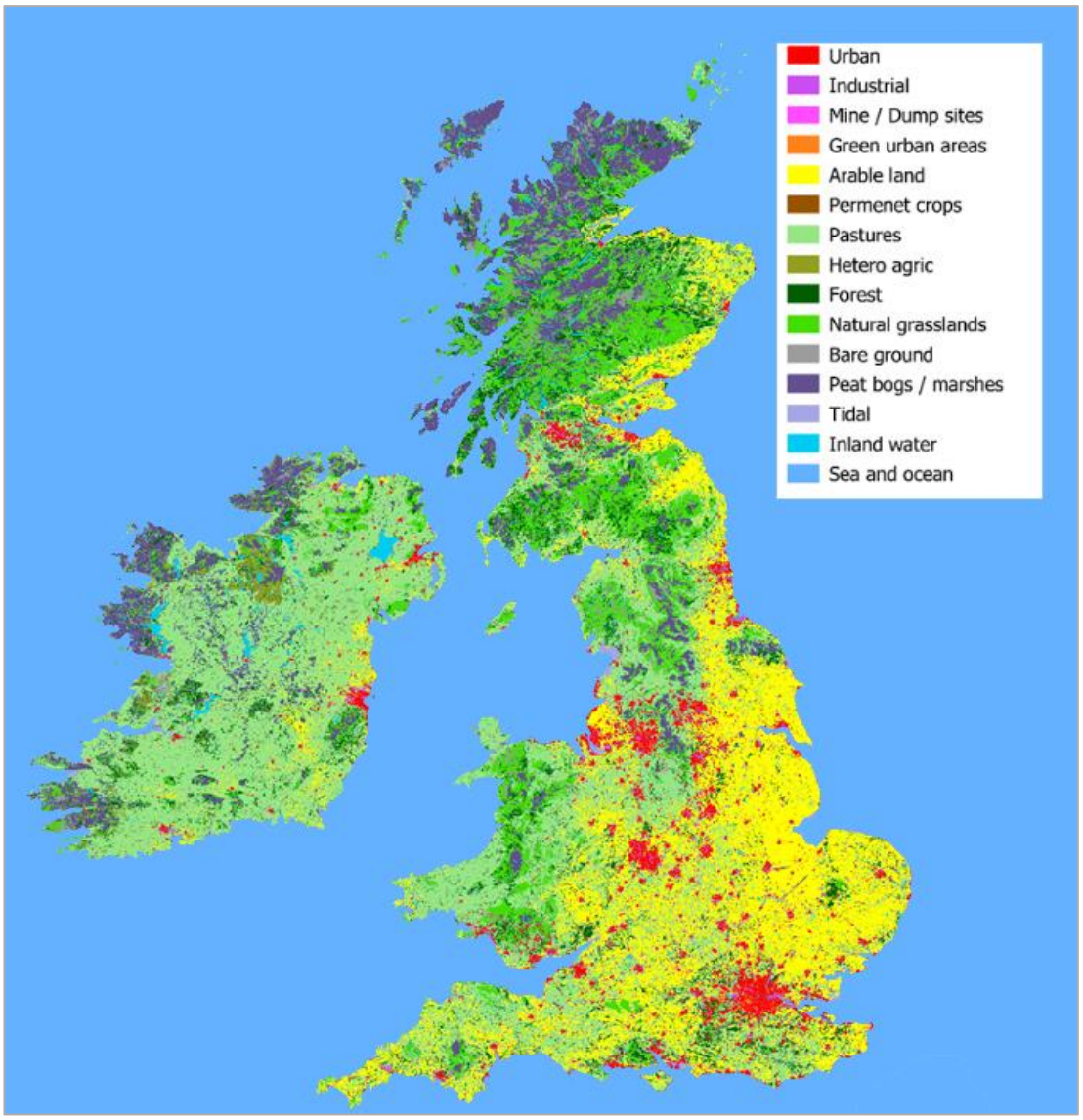

Figure 2.3. Land use in the British Isles based on the EU CORINE land 2018 dataset. There is a significant contrast in the land use of Britain (large areas of arable, notably in East England) and Ireland (predominantly pastoral).

The Marsh Fritillary only occurs where there is a relatively high abundance of pasture or coarse grassland, containing Devil's-bit Scabious. Ireland contains much more of this habitat than Britain. In Britain, the Marsh Fritillary has declined in both abundance and range, with its range now confined predominantly to the western part of the country. In contrast, in Ireland although it has probably suffered a reduction in numbers, the Marsh Fritillary retains much of its historic range. But, it is worth noting that the full historical range is poorly understood, Ford (1945) showed the species to be widespread across Ireland. This extent is reduced in recent maps, Asher *et al*.

(2001) identifies the counties of Fermanagh, Sligo, Donegal and west of the River Shannon as the chief of Ford's areas to still support the species in Ireland. This apparent decline from Ford's somewhat impressionistic distribution map is in part due to the lower recorder density and hence lower record number in the Republic of Ireland compared to the UK (Fox *et al*., 2010). This reflects a much more recent development of conservation interest in the Republic with Butterfly Conservation Ireland only founded in 2008 compared to 1968 for the UK Butterfly Conservation (Butterfly Conservation, 2018b). Therefore it is likely that the species is under-recorded, Barkham (2010) recounts the ease with which Maurice Hughes had found new Northern Irish colonies in the previous decade. As a result of this wider and less fragmented distribution, metapopulation dynamics and gene flow are likely to be far more intact in Ireland than in Britain and will possibly be similar to historical patterns.

The Irish distribution of the Marsh Fritillary is also broadly continuous across the island, as opposed to the disjunct distribution in Britain (Figure 2.1 D), with distinct northern and southern populations as identified by Joyce & Pullin (2003). This allows for the study of population differentiation over varying spatial scales. The spatial scale at which a study takes place as been shown to be important in butterfly species. An investigation of Mark Release Recapture (MRR) studies in the meadow brown (*Maniola jurtina*) found that the scale of the study was correlated to mean movement distance recorded, with increasing geographical scale also increasing the mean movement distance (Schneider, 2003). This demonstrates one of the limits of the MRR study system when used to investigate the movement and dispersal ability of a species, that it may underestimate the mobility of the target species.

Dispersal ability has long been known to limit a species ability to persist in an environment (Primack & Miao, 1992) and to colonise new areas of habitat (Papadopoulou *et al.*, 2009; Baselga *et al.*, 2012), dispersal ability is also important to the ability of a species to persist in a changing world (Travis *et al.*, 2013). Therefore,

it is important to understand the dispersal ability of a species undergoing active conservation efforts such as the Marsh Fritillary. Historically this has been done with MRR studies, however it has also been noted that MRR studies predominantly take place in altered landscapes, usually resulting from anthropogenic changes (Stevens *et al.,* 2010). In such an environment dispersal is expected to be more costly, usually in terms of increased mortality, than in an unaltered habitat, therefore such dispersals occur less frequently and so underestimations of dispersal ability may occur (Stevens *et al*., 2010).

Population genetics presents a powerful tool to help understand the dispersal ability of many species, including butterflies (Stevens *et al.*, 2010). It has been demonstrated that measures of population differentiation such as Fst are consistently related to dispersal ability across multiple animal taxa, with higher Fst values representing lower levels of dispersal (Bohonak, 1999). Thus, pairwise Fst values for sites can be used to estimate relative levels of movement between those sites (if the movement results in breeding).

The dispersal ecology of the Marsh Fritillary has not been heavily documented (Table 1.1). Dispersal distances have been studied and the maximum dispersal distance in the literature is 20km (Warren, 1994), however there is evidence of sex biased dispersal in the literature. Zimmermann *et al.* (2011) recorded that when long distance dispersals occurred in the species it was usually males who dispersed (>5km, 41 males:10 females; >10km, 13 males:1 female). This discrepancy may be explained by females mating soon after emergence and laying their first clutch in their natal patch, only possibly dispersing after this if sufficient food resources are available to produce a second clutch (Porter, 1981). Similar sex-biased dispersal has been observed in other butterfly species (Bennett *et al.*, 2013).

Given that the Marsh Fritillary is generally accepted to be a weak disperser (Table 1.1), it would be expected that isolation by distance (IBD) would be present in

the species. However results are currently conflicted with some studies finding evidence of IBD (Hula *et al.*, 2004) while others failed to do so (Joyce & Pullin, 2001). A reason for these contradictory findings could be spatial scale; Joyce & Pullin (2001) investigated populations across the UK while the study area of Hula *et al*. (2004) was less than ~450km^2. However it must be noted that a MRR study on the bog fritillary (*Boloria eunomia*) in four populations with varying levels of fragmentation showed a marked reduction in propensity to disperse, from 0.4 in the least fragmented populations to less than 0.05 in the most fragmented (Schtickzelle *et al.*, 2006). This could also be a factor in the Marsh Fritillary. Therefore, an investigation of the dispersal ability of the Marsh Fritillary butterfly using genetic techniques would be timely and to avoid some of the issues documented above it should take place over a wide variety of spatial scales and in a landscape with minimal anthropogenic fragmentation. Ireland presents an ideal study area as there are known Marsh Fritillary sites separated by a range of geographical scales and the species has not suffered any reduction in range which could have resulted in fragmentation.

The aim of this chapter is to identify the level and apportionment of genetic variation across Ireland using microsatellites. This will contribute to our understanding of the genetic variation of the species where it is stable and serve as a useful baseline for gauging the genetic health of declining populations. This is the first time a population genetics study of the Marsh Fritillary has been undertaken on this scale on a single land mass. Previous studies have either focused on smaller geographical areas (~450km^2, Hula *et al*., 2004; ~750km^2, Sigaard *et al*., 2008) or considered populations on different land masses (Britain and France, Smee, 2011). In contrast this study will assess the level of genetic variation and population differentiation in a single land mass in populations separated at different scales.

2.2 Method

2.2.1 Site selection and field sample collection.

Larvae were collected from ten sites across Ireland (Table 2.1 & Figure 2.4) in Autumn 2017 (MD), Spring 2018 (LW, LD) and Autumn 2018 (BC, BI, CW, DK, DV, LK, PK). Larvae from Northern Ireland (MD) were collected under Northern Ireland Department of Agriculture, Environment and Rural Affairs licence TSA/47/17. Sites were selected based on prior knowledge (from landowners, site managers and Butterfly Conservation Ireland) of the presence of healthy (not declining or very small) populations of the Marsh Fritillary. Selection was restricted to those sites where safe access and sampling permission could be secured from the land owner or site manager, due to legal requirements a licence was required to collect samples in Northern Ireland which further limited site selection. Of the subset of possible sites, sites to sample were selected to include varying degrees of geographical separation between the sites (Table 2.2). This includes three eastern sites very close, 11-12km, together (DK, LW & PK), three sites at a medium distance of separation, 33-43km (BI, CW & LK), two remote sites in the south (DV, 75km from nearest site) and north (MD, 97km) and an extremely remote site south west (BC, 193km).

All populations are assumed to be natural except for Bull Island (BI), this was first reported in 2011 however there is some doubt as to if this is a natural colonisation event as there are no other known sites in County Dublin (Harris *et al*., 2014). Marsh Fritillary larvae are easily identified in the spring and autumn as they form sibling webs, offspring of a single female. A single larva per web was collected to prevent the collection of closely related individuals. A maximum of 30 larvae per site were collected as this was judged to give a fair representation of the genetic diversity at each site (Hale *et al*., 2012; Smee, 2011).

Table 2.1 Site information for Ireland. Surrounding land use taken from EU CORINE land use data set, underlined land use is land use of site, other listed are land uses of surrounding area. Geological information form British Geological Society and Geological Survey Ireland.

Location	Code	Location	Elevation (m)	Site description	Surrounding land use	Bedrock geology	Sediment geology
Barleycove, Co. Cork	BC	51.46774 / -9.78179	47.9	Coastal, sloping roadside field.	Pastures, Moors and heathland, Beaches, dunes, sands	Old Red Sandstone; sandstone, conglomerate & mudstone	Till derived from limestone
Bull Island, Dublin	BI	53.37453 / -6.13584	3	low-lying flat rough grassland	Sport and leisure facilities, Beaches, dunes, sands, Intertidal flats, Salt marshes	Viséan limestone & calcareous mudstone	Wind-blown sand
Mullingar Co. Westmeath	CW	53.5114 / -7.2726	94	Pasture land beside a small river	Pastures, Transitional woodland-shrub, Peat bogs, Discontinuous urban fabric	Tournaisian limestone	Peat, till derived from limestone
Dunshane Common Co. Kildare	DK	53.15244 / -6.67216	123.2	Large hedged pasture field	Pastures, Non-irrigated arable land, Natural grasslands, Transitional woodland-shrub	Viséan limestone & calcareous mudstone	Till derived from limestone

Derryvilla, Co. Tipperary	DV	52.79967 / -7.81471	108.8	Flat pasture lands with low hedges	Pastures, Discontinuous urban fabric, Non-irrigated arable land	Tournaisian limestone / Viséan limestone & calcareous mudstone	Till derived from limestone
Lullybeg Co. Kildare	LK	53.29203 / -7.61578	54.6	Pasture land with low hedges and occasional small trees	Pastures, Non-irrigated arable land, Transitional woodland-shrub	Tournaisian sandstone, mudstone, limestone	Alluvium, till derived from limestone
Lullymore Co. Kildare	LW	53.269 / -6.94251	79.6	rough grassland surrounded on three sides by small	Transitional woodland-shrub, Pastures, Peat bogs, Discontinuous urban fabric	Tournaisian limestone	Peat, till derived from limestone
Murlough Dunes, Co. Down	MD	54.23678 / -5.85477	13.1	Rough grassland, possibly former pasture land, close to coast	Natural grasslands, Non-irrigated arable land, Intertidal flats, Estuaries, Sport and leisure facilities	Late Ordovician to Silurian greywacke, mudstone	Wind-blown sand

Pollardstown Fen Co. Kildare	PK	53.1836 / -6.84568	91	Pasture and rough ground with heading and a few trees	Pastures, Non-irrigated arable land, Inland marshes	Siluro-Devonian granitic rocks & appinite	Peat, till derived from Devonian sandstone
Lough Derravaragh, Co. Westmeath	LD	53.66709 / -7.37819	61.5	Damp rough grassland on edge of water	Inland marshes, Pastures, Water bodies	Viséan limestone & calcareous mudstone	Peat

Regional climate data for the counties from which samples were collected (sites within that county are listed). Data given for each county: maximum average annual temperature (°C), minimum annual temperature (°C), total rainfall (mm), respectively. Co. Cork (BC), 13.2°C, 7.9°C, 727.9mm. Co. Westmeath (CW, LD), 12.9°C, 5.7°C, 941.3mm. Co. Kildare (DK, LK, PK, LW) 13.4°C, 6.1°C, 754.2mm. Co. Tipperary (DV), 14.0°C, 7.4°C, 977.6mm. Co. Down (MD), 12.9°C, 5.3°C, 1143.7mm. Dublin (BI), 13.3°C, 6.4°C, 758mm (for detailed climatic data see Appendix C).

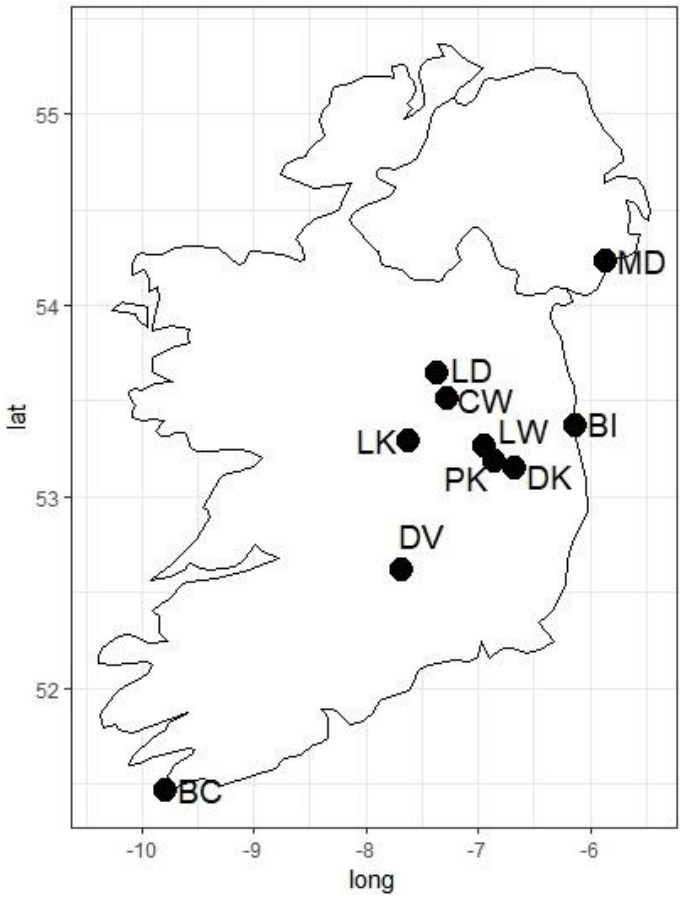

Figure 2.4. Site locations included in this study, for site codes see Table 2.1

Table 2.2. Distance (km) between site pairs. Highlighted distances indicate the shortest distance for that row. Classification of sites: *close, **intermediate, ***remote, ****extremely remote.

	BC	BI	CW	DK	DV	LK	LW	MD	PK
BC**	-	326	284	283	193	251	278	406	277
BI**	326	-	77	43	133	99	55	97	52
CW**	284	77	-	57	103	33	35	123	46
DK*	283	43	57	-	90	65	22	132	12
DV***	193	133	103	90	-	75	88	217	84
LK**	251	99	33	65	75	-	45	156	53
LW*	278	55	35	22	88	45	-	129	11
MD***	406	97	123	132	217	156	129	-	134
PK*	277	52	46	12	84	53	11	134	-

2.2.2 Laboritary work and analysis

Specimens were stored at -80°C until used. Larval heads were used as the source and DNA extracted using the Livak method (Livak, 1984). Microsatellites developed by Smee *et al*. (2013) were used (Aurinia_01, Aurinia_13, Aurinia_16 & Aurinia_64), these were amplified using the conditions described by Smee *et al* (2013), with an increase in annealing temperature of 2°C for Aurinia_16 (full details see Appendix A). PCR products were separated via capillary electrophoreses using an AB3500 Genetic Analyser (Applied Biosystems) and sized relative to the LIZ500 (Applied Biosystems) internal size standard using GeneMapper 5. Sizes were checked manually and individuals with unclear peaks were reamplified and re-genotyped. Raw allele scores were binned using TANDEM to reduce error in the binning process.

Analysis was carried out in R (version 3.3.2) using binned allele sizes unless otherwise stated, no transformations of the data was carried out unless otherwise stated. To characterise the genetic variation within populations basic population statistics were calculated using `diveRsity` (Keenan *et al*., 2013). Pairwise Fst and Nei's G'st were calculated with corresponding p-values were calculated using a permutation test (10,000 replicates) in `strataG` (Archer *et al.*, 2017) to determine the level of differentiation between populations. Isolation by distance (IBD) was tested using distance-based redundancy analysis (dbRDA (Meirmans, 2015)) this was carried out on a table of pairwise Fst values which was constrained by the geographical XY locations of the samples in order to calculate the proportion of the variation explained by location. This was done using `vegan` (Oksanen *et al*., 2019).

Spatial Principal Component Analysis (sPCA) were carried out using package `adegenet` (Jombart, 2008) to investigate spatial patterns of genetic variability. The principal component scores of the allele frequencies of an individual are multiplied by Moran's *I* which is a measure of spatial autocorrelation a Monte Carlo permutation test is then performed to test for global and local structuring (the tendency of

neighbouring sites to be similar and dissimilar, respectively). Where there is global structuring neighbours are more likely to be genetically similar and there is a high degree of positive autocorrelation, local structuring indicates genetic dissimilarity among neighbours and negative autocorrelation (Warren et al., 2016). Eigenvalues from the sPCA were examined to determine how many axes should be retained. The sPCA was visualised as a 3-colour plot using package `adegenet` (Jombart, 2008) with `raster` (Hijmans, 2019) and `rgdal` (Bivand *et al*., 2018) used to provide the geographical base layer to the plot. The lagged scores were used for this as they reduced the 'noise' in the data making them better for identifying global structuring. The red/green/blue bands used to define colour on computer graphics are assigned to the lagged scores for each of the first three principal components such that similarity of colour indicates genetic similarity.

The statistical power of the microsatellites used was assessed in POWSIM 4.1 (Ryman & Palm, 2006), this used allele frequencies for the total dataset to carry out a Fisher's exact test with 10000 replicates. The presence of null alleles checked using FreeNA (Chapuis & Estoup, 2007).

2.3 Results

A single sample was collected from site LD therefore this site was removed from further analysis. All other sites had between 18 and 30 samples ($\bar{x}$=24.7).

Power analysis showed that, based on detected allele frequencies and under a conservative estimate of an effective population size of 2000 individuals (assuming all samples are unrelated and taking into account field observations), the microsatellites used would be able to detect Fst of 0.01 in 89% of cases and Fst of 0.02 in all cases which was deemed sufficient to answer the research question.

Analysis suggests that null alleles may be present in six out of nine populations for either Aurinia_16 (DV & LW) or for A urinia_16 and A urinia_64 (BC, BI, CW, PK). There was no evidence of null alleles in DK, LK and MD.

For populations where the sample size >1, allele number varies between 12 and 18 (mean = 15.11), and allelic richness lies between 2.29 and 3.40 (mean = 2.93) (Table 2.3). Private alleles are absent at five of the nine sites (CW, DK, LK, MD and PK) and are present at four, varying from 5.56% (BI) to 16.67% (DV). H_e is fairly consistent across all sites (0.43 – 0.57, mean = 0.50, overall =0.57) and H_o varies from 0.30 at BI to 0.55 at LK (mean = 0.43, over all = 0.42).

Table 2.3. Sample sites in Ireland, note that as only one sample was collected from LD it was not included in further analysis. Total values are the totals across all sites except for allele richness which is a mean across all sites. Allelic richness is calculated using 1000 resamples (n=smallest input sample size), with replacement per population, and the mean value across all loci is give. Private alleles are given as the percentage of the total alleles across all loci found only in that population. H_e is average expected heterozygosity across all loci. H_o average observed heterozygosity across all loci. Fishers exact test with 1000 iterations was performed to detect significant departures from Hardy–Weinberg equilibrium (HWE).

Site	Sample size	Allelic richness	Private alleles (%)	H_e	H_o	HWE	F_{IS} (95% CI)
BC	30	2.84	14.29	0.47	0.36	<0.001	0.234 (0.053, 0.414)
BI	30	3.14	5.56	0.49	0.30	<0.001	0.396 (0.396, 0.511)
CW	30	2.88	0	0.56	0.45	<0.01	0.192 (0.031, 0.353)
DK	23	3.03	0	0.44	0.45	<0.001	-0.037 (-0.231, 0.170)
DV	10	2.66	16.67	0.43	0.44	0.054	-0.015 (-0.365, 0.355)
LK	28	2.86	0	0.53	0.55	<0.001	-0.036 (-0.223, 0.133)
LW	23	3.40	5.89	0.57	0.51	<0.001	0.104 (-0.051, 0.254)
MD	18	2.29	0	0.50	0.49	<0.05	0.001 (-0.147, 0.150)
PK	30	3.29	0	0.50	0.32	<0.001	0.352 (0.191, 0.531)
LD	1	-	0	-	-	-	-
Total	222	2.93		0.57	0.42	<0.001	0.266 (0.211, 0.317)

Table 2.4. Pairwise Fst (below the line) and G`st (above the line) for each population. Significant scores are denoted as follows: * p<0.05; ** p<0.01; *** p<0.001. Overall Fst 0.1141 (p<0.01), overall Gst 0.1213 (p<0.01), n=221.

	BC (n=30)	BI (n=30)	CW (n=30)	DK (n=23)	DV (n=10)	LK (n=28)	LW (n=23)	MD (n=18)	PK (n=30)
BC	-	0.1344***	0.1265***	0.1629***	0.1324***	0.1651***	0.1726***	0.0808**	0.1155***
BI	0.1386***	-	0.1374***	0.0560**	0.1974***	0.0494***	0.1147***	0.1483***	0.0930***
CW	0.1303***	0.1451***	-	0.1437***	0.1031***	0.1128***	0.1563***	0.0293*	0.0784***
DK	0.1631***	0.0625**	0.1453***	-	0.1502***	0.0243*	0.0797***	0.1246***	0.0567**
DV	0.1313***	0.2008***	0.1072***	0.1541***	-	0.1718***	0.1184**	0.0606**	0.1177***
LK	0.1671***	0.0583***	0.1185***	0.0293*	0.1725***	-	0.0747***	0.1216***	0.0726***
LW	0.1732***	0.1233***	0.1619***	0.0827***	0.1228**	0.0811***	-	0.0933***	0.0658***
MD	0.0827**	0.1548***	0.0374*	0.1290***	0.068**	0.1263***	0.0975***	-	0.0469**
PK	0.1169***	0.1014***	0.0856***	0.0592**	0.1212***	0.0785***	0.072***	0.0522**	-

All but one population show significant departures from HWE, the exception is DV. MD and CW show lower, though still significant departures from HWE than the other populations. There is variation in the inbreeding coefficient, ranging from -0.037 to 0.396, however there is no evidence of significant levels of inbreeding (Table 2.1). Overall Fst and G'st reveal significant levels of population structuring in Ireland (0.114 and 0.1213 respectively, both $p<0.01$, $n=222$), with significant pairwise Fst and G'st between all populations in the study (Table 2.4).

Spatial Principal Component Analysis (sPCA) also revealed significantly high levels of global population structure within the data set ($p<0.001$, $\lambda=0.263$, Figure 2.5) but no significant local structuring ($p>0.05$, $\lambda=0.021$).

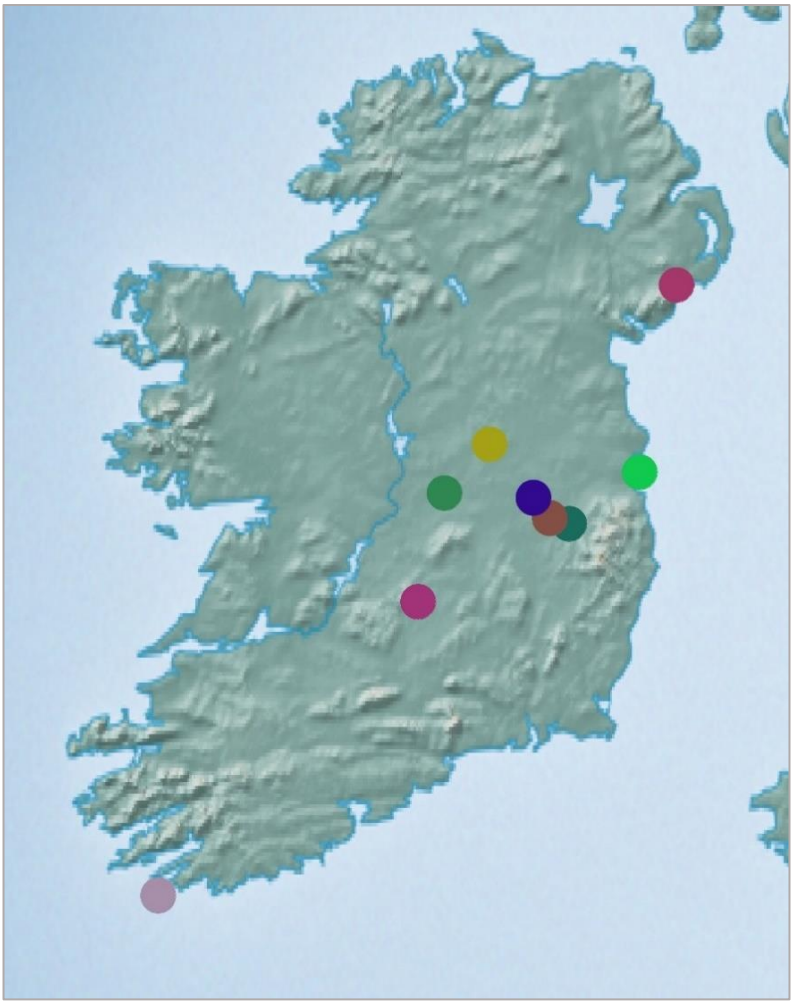

Figure 2.5. Colorplot of sPCA for all Irish sites (except for LD). The colours red, green and blue are assigned to the lagged scores of the first three principal components, respectively. Similarity of colour indicates genetic similarity

Isolation by distance was confirmed, geographic location accounted for 30% of the variance in the dataset ($Adjr^2$=0.30, F=2.74, p<0.05, df=2,6 Figure 2.6). Latitude was found to be significant (F=3.48, p<0.05, df=1,6) but longitude was not (F=2.01, p=0.13, df=1,6). The notable outlier in Figure 2.6 is the MD-BC pairing and is suspected to be due to homoplasy.

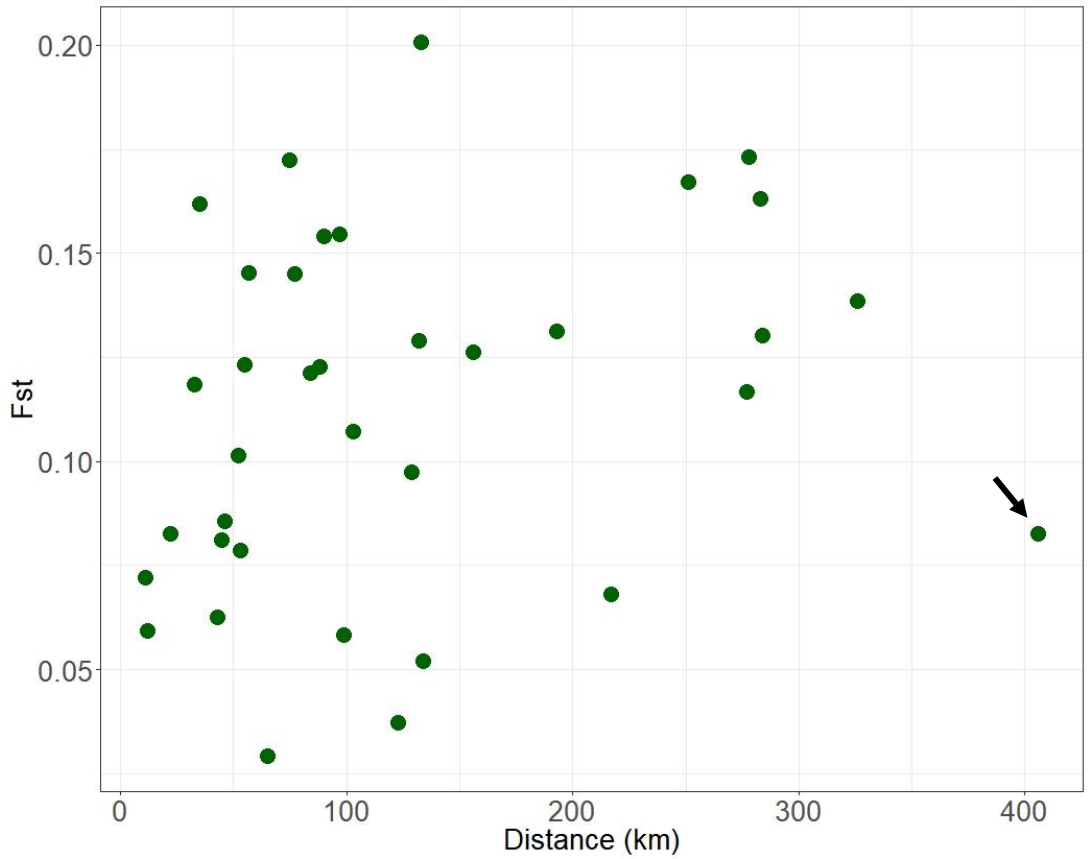

Figure 2.6. Ireland shows evidence of IBD, site pairs with a greater distance generally have a higher Fst, G'st displays a similar pattern (data not shown). The notable outlier is MD-BC (arrow).

2.4 Discussion

This study reveals populations in Ireland that show significant deviation from HWE and exhibit a high degree of population differentiation across varying geographic scales. The majority of populations contained a significant deficiency of heterozygotes. This has been reported in several other studies on Lepidoptera (Vila

et al., 2006; Orsini *et al*., 2008; Pecsenye *et al*., 2017). The presence of null alleles may explain the departures from HWE, although they were only reported in six of the nine populations (BC, BI, CW, DV, LW & PK): three other populations (DK, LK & MD) also show departures from HWE but do not show any evidence of null alleles. Hence the presence of null alleles cannot fully explain the observed departures from HWE.

Two of those populations (LK and DK) demonstrate an excess of heterozygotes. This may be caused by non-assortative mating. During the final instars the larvae, which have previously remained in larval webs formed of siblings, disperse and are completely solitary during the sixth instar and pupation. This reduces the likelihood of mating with close relatives and promotes disassortative mating. In contrast, the MD population shows a heterozygote deficiency, the most likely cause being linked to genetic bottlenecks following population fluctuations which are known to have occurred at this site (Joyce & Pullin, 2001). Such reductions in genetic diversity following a population bottleneck has been demonstrated in other lepidoptera species (Jangjoo *et al.*, 2016) as well as many other species (Xenikoudakis *et al.*, 2015; Abascal *et al.*, 2016)

The occurrence of isolation by distance (IBD) was in contrast to the findings of Joyce & Pullin (2001, 2003) who recorded no IBD in the species in the UK. However their study utilised a Mantel test to assess IBD and the suitability of this approach has since been brought into question (Guillot & Rousset, 2013). Instead approaches such as the distance-based RDA used here are recommended due to the increased statistical power (Meirmans, 2015). The finding of IBD is consistent with the ecology of the Marsh Fritillary as it is considered to be a relatively weak disperser (Dennis & Hardy, 2018). Dispersal and colonisation of 10-20km has been recorded (Warren, 1994; Zimmermann *et al*., 2011), but this is still a short distance compared to the size of Ireland (~84,000km^2) and the distance between sites (>400km).

An interesting finding is the much lower than expected Fst/G'st pairwise values for MD-BC and the similarity of colour for these two sites on the colour plot. These two sites are the most distant in the study (406km apart) and yet have lower Fst and G'st values than between BC and its nearest neighbour DV. Frequent dispersals between MD and BC, which avoid all intermediate sites, are unlikely to the point of being all but impossible. Therefore, the most likely explanation is homoplasy. The microsatellite allele at the two sites are identical by state but not by descent. This can be expected to occur due to the high mutation rate of microsatellites and the allele size constraints (Jarne & Lagoda, 1996; Viard *et al*., 1998; Estoup *et al*., 2002; Bhargava & Fuentes, 2010; Putman & Carbone, 2014). This could be confirmed by the application of alternative techniques such as microsatellite genotyping by sequence or the use of single nucleotide polymorphisms (SNPs) (Coates *et al*., 2011; Vartia *et al*., 2016).

Comparable studies on the population genetics of the Marsh Fritillary are rare. Joyce & Pullin (2001) used allozymes to investigate the population genetics of the Marsh Fritillary in the UK and France, including a single population from Northern Ireland, but none from the Republic of Ireland. Thus, due to the lack of samples from other sites on the same landmass, the significantly high pairwise Fst values reported for the Northern Ireland population compared with all others in the study is hardly surprising. Hence it is impossible to say if that result is representative of the general situation within Ireland. However, the pairwise Fst values obtained in this study are of a similar range to those reported by Joyce & Pullin for samples within Britain (0.0293 – 0.2008 and 0 - 0.2819, respectively) and for pairs including France (0.0363 – 0.1644) suggesting that the Northern Ireland popuatlion used by Joyce and Pullin is representative of the landmass as a whole and therefore can be used as a proxy for pairwise comparisons between the British and Irish landmasses.

Comparisons with other Lepidoptera species must be with either closely related species or, where this is not possible, with species that have similar life history traits. *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), though a pest species of fruit orchards (Higbee *et al*., 2001), has a similar life history and ecology to the Marsh Fritillary in terms of host plant specificity, life cycle and population structure. Swiss populations exhibited comparable levels of population differentiation even between sites within 10km of each other (Chen & Dorn (2010). In contrast, in another comparable species, *Coenonympha hero* (L.) (Lepidoptera: Nymphalidae), population differentiation was largely related to the extent of geographic separation and degree of isolation, although peripheral but connected populations had a higher degree of population differentiation compared with those which were fully isolated (Cassel & Tammaru, 2003). This suggests that the factors affecting levels of population differentiation are highly species specific and substituting data from another species, based on similarity of ecological traits, is inadvisable.

With the widespread maintenance of pastoral land use in Ireland and the extensive distribution of the larval food plant, it is tempting to view the Irish landmass as being occupied by adjacent metapopulations of Marsh Fritillary, with only short dispersal distances separating metapopulations. Thus, alleles can move slowly across the landscape and this gene flow unites populations. However, the significant level of population differentiation even across relatively small distances suggests this view is misguided and gene flow is scarce. Even the three sites which are all within 25km of each other (LW, PK and DK) have significant pairwise Fst values, reflected in the three sites being represented by three distinct colours in the sPCA plot, indicating limited gene flow between them. A similar pattern is seen in the brush-tailed rock-wallaby (*Petrogale penicillata*) in Queensland, Australia, where significant levels of population structuring was detected in continuous habitat (Hazlitt *et al.*, 2006). As with the Marsh Fritillary, significant population differentiation occurred over distances

of <10km and it is suggested that this lack of dispersal may have contributed to the threatened status of the brush-tailed rock-wallaby.

Work based on the COI gene suggests that the Irish populations form a unique lineage within Europe (Whitla, 2019). The paucity of private alleles revealed in this study implies that populations are interconnected but at a rate too low to offset the rate of population differentiation resulting from genetic drift and bottlenecks produced by highly fluctuating numbers within individual populations (Slatkin, 1985; Gompert *et al.*, 2014; Pecsenye *et al.*, 2017).

The natural boom-bust population cycles have been studied in various butterfly species (Thomas & Hanski, 1997) and has been well documented in the Marsh Fritillary (Ford & Ford, 1930; Donovan, 1936). Donovan, quoting a letter from Sir Charles Langham, described a field in Enniskillen in 1928 'It would be an exaggeration to say the field was black with them but not very far from the truth'. E.B. Ford (1945) noted how the Cumbrian population he and his father studied from 1881 increased to become very abundant in 1894 until it began to decline in 1897. By 1913 is was very rare, a pattern which persisted until 1919 before increasing again from 1920 to 1926 when it remained abundant until the study ceased in 1935. Within this natural cycle of approximately 45 years there were perhaps only three years when wider dispersal was likely to occur (Donovan, 1936). Indeed Ford (1945) remarked that the individuals rarely left the site.

Mark-release-recapture (MRR) assessments of dispersal distances vary (Table 1.1). Junker & Schmitt (2010) recorded a mean of under 100m, with a few individuals moving greater than 300m. Other studies have suggested distances of 2-3km (Fric *et al*., 2010; Konvicka *et al*., 2012), while Zimmermann *et al*., (2011) and Warren (1994) found dispersal distances of 10km and 15-20km respectively. When these studies are taken together, alongside the limitation of MRR studies (e.g. that marked individuals who move outside of the study area are not recaptured) it can be

assumed that short dispersal distances are most common but dispersal over longer distances (>5km) occurs often enough to merit consideration when investigating gene flow. Previous studies of gene flow via molecular methods have determined maximum dispersal distances of up to10-14km (Sigaard *et al*., 2008).

The distances between some of the sampled populations (11-12km) are within the upper end of the range of published dispersal distances (Table 1.1) and yet there is significant levels of population differentiation. This restricted gene flow even between the geographically closest populations is akin to those recorded in Danish populations (Sigaard *et al*., 2008). Therefore, there must be some process preventing or limiting gene flow between populations. Given that microsatellites are selectively neutral it is highly unlikely that local adaptation is driving the population differentiation. Some other reason, based upon HWE conditions, must be sought.

The cycles of population decline and expansion found in the species will result in genetic bottlenecks, when combined with weak dispersal, these may be responsible for the patterns of population differentiation observed in Ireland. As a general rule it is considered that one migrant per generation (OMPG) is sufficient to maintain gene flow and prevent the effects of drift. However this is a theoretical model based on an ideal population with unrealistic assumptions such as an infinite number of sub populations, absence of selection and no geographical structuring (Wright, 1969). There have been attempts to develop corrections for some of the violations that result from the application of OMPG to real populations (Wang, 2004). It has been suggested that between 1 and 10 migrants per generation are required (Mills & Allendorf, 2003) but under certain demographic and life history scenarios less than 1 migrant per generation is sufficient to prevent population differentiation (Wang, 2004).

The long-term study of the Glanville Fritillary, *Melitaea cinxia* (L.) in Åland Islands, Finland, has shown that strong genetic spatial structuring occurs at a local level (<15km) as well as at a landscape level (Saccheri *et al*., 2004). In addition

frequent extinctions and recolonisation promote population differentiation as does increasing distances between population pairs (Saccheri *et al*., 2004). This is very close to what has been recorded here in Ireland for the Marsh Fritillary and it is likely that similar underlying processes are at work.

It is also possible that the Irish landscape, despite its pastoral mosaic, is less connected than it first appears to be. The presence of adjacent populations within the dispersal distance of the Marsh Fritillary found in this study suggests the classic metapopulation structure is still in place, at least in some of Ireland. Nevertheless populations are threatened by land use change (Fox *et al*., 2006) which may result in a change from a classic metapopulation structure to a disequilibrium structure (Harrison & Taylor, 1997). This is characterised by sub-population extinction without the chance of subsequent recolonisation. This may already have occurred in some areas, although it is possible that current patterns of genetic variation do indeed reflect historical ones.

Sang *et al*. (2010) reported evidence of extinction debt in calcareous grassland butterflies 50 years after habitat changes and Soga & Koike (2013) found extinction debt among specialist butterflies in Tokyo following 40 years of rapid urban development. In contrast Krauss *et al*. (2010) found that after 40 years the extinction debt had been paid in grassland butterflies though it persisted in the plant species. This suggests that it is possible for butterfly species to persist with an extinction debt for 30-50 years. The rapid declines of the Marsh Fritillary seen in Britain in the late-20th and early-21st century was, at least in part, the payment of the extinction debt owed from the rapid land use changes which may have shifted the local populations into non-equilibrium metapopulations with individual populations persisting for several decades before going extinct.

Genetic extinction debt, the future loss of genetic diversity due to past environmental changes, has been little explored (Plue *et al*., 2017). Most studies of

this idea have utilised plants, although these are difficult to compare with invertebrates, due to either their longevity (eg trees; Vranckx *et al*., 2012) or the presence of alleles from previous generations via a seed bank (Plue *et al*., 2017). When studies have taken place on animals, they have been charismatic mammals (Smith *et al.*, 2018). In cases where genetic extinction debt has been detected it is characterised by a pattern of high total genetic diversity combined with low population differentiation (Vranckx *et al.*, 2012; Plue *et al.*, 2017; Smith *et al.*, 2018). This pattern is not observed in this study, Irish populations of Marsh Fritillary display high population differentiation. This suggests that a genetic extinction debt is unlikely to be owed in Ireland.

Ireland is on the westernmost edge of the range of *E. aurinia*. Based on the variation observed in this study the region may be of notable conservation value due to the degree of population genetic differentiation (Lesica & Allendorf, 1995; Glass *et al*., 2015; Steen & Barrett, 2015). It also provides evidence for a higher degree of natural population differentiation than might otherwise be assumed based on similar and related species. The lack of evidence for an extinction debt implies that Ireland is suitable to act as an example of the historical condition and as a suitable benchmark for the natural, undisturbed population genetics of the species.

3 By Land and By Sea: The effect of island distribution on population differentiation in the Marsh Fritillary butterfly.

3.1 Introduction

Biogeography was originally the study of the geographical distribution of species (Brown, 1983). It has subsequently expanded to include any variation in biological features (including phenotypic, genetic or behavioural) across geographic scales (Brown & Lomolino, 2006). Much of the biogeographic research has focused upon the effect of the isolation of populations through natural features. This has become known as island biogeography (Lomolino *et al*., 2010). Although oceanic islands and archipelagos are typically first to mind when considering island biogeography, the theory can be applied to a variety of fragmented habitats including mountain tops, dung piles and protected areas. (Wu & Vankat, 1995).

In applying island biogeography theory to situations other than oceanic islands, the landscape is viewed as patches of suitable habitat ('islands') in a matrix ('sea') of unsuitable habitat (Haila, 2007). To assess the ability of a given species to colonise or migrate to a new island, the distance from the inhabited patch to the new island must be considered (Littlewood *et al*., 2009), along with the nature of the matrix and the specific challenges and dangers that it presents (Koh & Ghazoul, 2010).

All species have a maximum dispersal distance. When patches are separated by a distance greater than this movement between them is impossible (Baguette *et al*., 2000; Watson *et al*., 2005). In addition the permeability and quality of matrix habitats varies, with highly permeable, high quality habitats presenting less of a barrier to dispersal than low quality habitats with low permeability (Åström & Pärt, 2013; Evans *et al*., 2017). Since movement of individuals can also result in gene flow if those individuals reproduce in the new patch, patches which are closer together or where

the matrix is highly permeable will experience greater gene flow than those patches which are a greater distance apart or where the matrix is of low permeability.

Metapopulation theory shares many traits with island biogeography concepts (Hanski & Glipin, 1991). However a key difference is that while island biogeography is often traditionally concerned with communities, metapopulation theory focuses on the dynamics of a single species (Hanski & Simberloff, 1997). Since the persistence of a metapopulation requires the movement of individuals to colonise new patches or to recolonise patches where populations have become extinct the effect of patch distance and matrix permeability is critical to the long-term persistence of the metapopulation (Ferreras, 2001; Johst *et al*., 2002; Driscoll *et al*., 2013).

When a metapopulation potentially exists across several islands it presents an additional complexity. Movements between patches on a single island may be assumed to occur with the frequency that they would between patches of a similar distance on the mainland. However, it is also reasonable to assume that the open water presents a lower quality and less permeable matrix than a similar distance in a terrestrial matrix (Jha, 2015). An open water matrix lacks the resting and potential feeding sites that may reasonably be expected to occur in a terrestrial matrix even when the matrix does not contain habitat suitable for long term occupancy or breeding. Therefore, it would be expected that movement, and consequently gene flow, would be more restricted when patches are separated by water than when they are separated by terrestrial non-habitat.

Most studies on the effect of island distribution on species have focused on the extinction/colonisation dynamic or factors influencing the occurrence of a species on an island, where the genetics of an island distribution has been considered it has predominantly been from a phylogeographic perspective (Santos *et al*., 2016). However where studies have looked at population structure the results have been conflicting as to the effect of an island distribution on population structure, suggesting

that it is highly species specific (Koh *et al*., 2002). A positive relationship between increasing overwater travel distance and Fst has been found in the wasp species *Polistes dominulus* (Dapporto *et al.*, 2009) while significant population structuring was absent in non-migratory populations of the Monarch butterfly (*Danaus plexippus*) on the Hawaiian islands (Pierce *et al.,* 2014). The Monarch butterfly is known to be highly dispersive, however significant levels of population structuring have been detected between island populations of less dispersive butterfly species (Baxter *et al.*, 2017), confirming that response to island distribution is species specific.

The Marsh Fritillary butterfly is a metapopulation species with a broad, though declining, European distribution. This decline is also occurring generally within the British Isles, however in western Scotland populations are stable and even expanding (Fox *et al*., 2010; RSPB, pers. comm). In Scotland it is found both on the mainland in the Argyll region and on several of the islands of the Inner Hebrides (Figure 3.1). The dispersal ability of the Marsh Fritillary is debated but the maximum dispersal distance is generally accepted to be around 15km based on Mark-release-recapture (MRR) and gene flow studies (Table 1.1; Warren, 1994; Sigaard *et al*., 2008; Zimmermann *et al*., 2011). However, these studies were all undertaken on either mainland sites, or a mainland-island site with less than 1km (at narrowest point) of water separating them (Sigaard *et al*., 2008). In contrast some of the Inner Hebridean Islands inhabited by the Marsh Fritillary are separated by open water distances in excess of 15km.

As the distance between islands and the islands and the mainland in the Hebrides varies the level of migration and thus gene flow between these populations is potentially complex. The hypothetical gene flow patterns are that:

1. The open water presents no barrier, population differentiation between the island population is similar to the differentiation seen between mainland sites of an equivalent separation distance.

2. Butterflies are unable to cross open water; population differentiation is total, or greatly exceeds that of mainland populations given equivalent distances apart.

3. Movement is in a stepping stone fashion, differentiation is least between the sites that are separated by the shortest distance across open water and greatest where there is a larger open water distance.

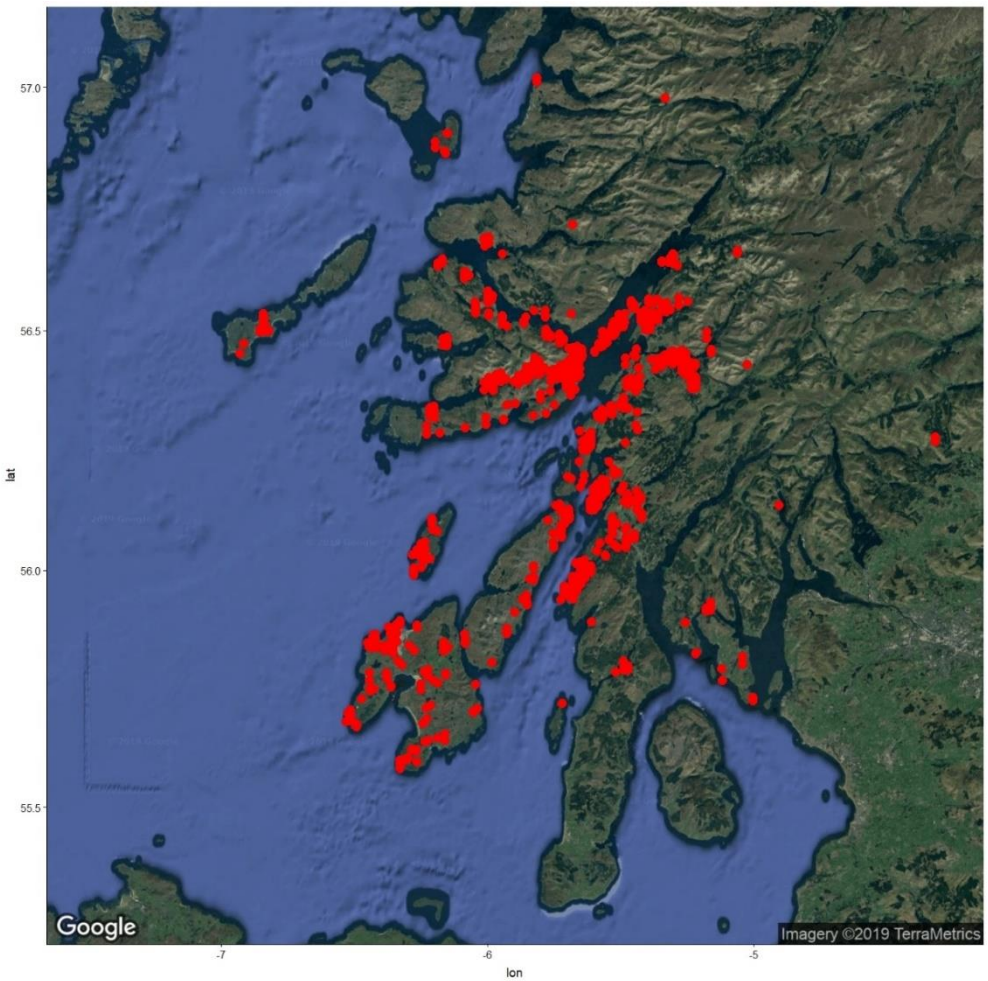

Figure 3.1. Post-2000 distribution of the Marsh Fritillary in Western Scotland and the Inner Hebrides. It is important to note that due to low human population density in this region it is likely that the Marsh Fritillary is under recorded. Points represent records of occurrence; one point may represent multiple records at the same geographical location. Combined datasets from NBN Atlas (2019) & GBIF (2019).

The mobility of butterfly species in the British Isles has been characterised by Dennis & Hardy (2018) based upon rank sum values for the following variables:

1. Vagrants recorded in ex-habitat.
2. Suburban garden records.
3. Central urban records, for example from business districts of major population centres such as inner-London, Birmingham and Sheffield.
4. Range expansions.
5. Mass movements of individuals.
6. At-sea records or documented records of sea crossings
7. Recorded sea crossing >10km.
8. Frequent long-distance reversed mass migrations.
9. Over-ocean movements.

Based upon this scale Dennis & Hardy assessed the Marsh Fritillary as having a Mobility Index of 7 out of 45 and state that there are no records of sea crossing for the Marsh Fritillary and no occurrence of crossing to an island across more than 10km of open water. Based on this assessment the Marsh Fritillary should be incapable of crossing between at least some of the Inner Hebridean islands.

However, the Marsh Fritillary is found in Ireland and accepted to have colonised the island from Britain either via Scotland (Junker *et al.*, 2015) or via Wales and south England (Joyce & Pullin, 2001). The minimum open water distance between the landmasses is ~30km at its narrowest, clearly in excess of the 10km stated by Dennis & Hardy (2018). The possibility of a land bridge between Britain and Ireland has been largely regarded as unlikely, of short duration if it did occur and the species crossing it would be restricted to those which were cold tolerant (Edwards &

Brooks, 2008). Phylogeographic data suggests that relatively few individuals were involved in establishing the Marsh Fritillary in Ireland, both Joyce & Pullin (2001) and Whitla (2019) report no haplotype variation in Ireland compared to multiple haplotypes in Britain. This suggests that the colonisation occurred either over a brief time period (if the Marsh Fritillary was able to utilise the hypothetical land bridge) or as the chance movement of individuals beyond their usual dispersal distance. Barnett & Warren (1995) report the occurrence of a Marsh Fritillary colony at a patch >20km from another known colony, although it is acknowledged that this could be a clandestine release, it could also suggest that dispersal over distances comparable to the Irish sea, which may have been narrower at the time of colonisation, may occur though perhaps very infrequently.

Despite this apparent lack of colonisation ability, it is known that the Marsh Fritillary populations in the Inner Hebrides are stable and even expanding (RSPB, pers. comm.). This stability, when so many other populations throughout the species range are declining, provides an opportunity to study dispersal amongst islands.

The Inner Hebrides is an archipelago of 79 islands (35 inhabited), off the west coast of Scotland which extends for approximately 240km from Skye in the north to Islay in the south. This study is based in the southern Hebrides, this extends as far north as Mull covering approximately 125km, north to south. The most westerly island is Tiree, approximately 85km from the mainland. The majority of the landscape is dominated by grassland, some of which is lightly grazed by cattle or sheep. In addition, some islands, notably Islay and Jura, are dominated by peat bogs which are found throughout the area (Figure 3.2). The larval food plant for the Marsh Fritillary Devils-bit scabious) is found throughout the region, indicating ample potential habitat patches (Figure 3.3).

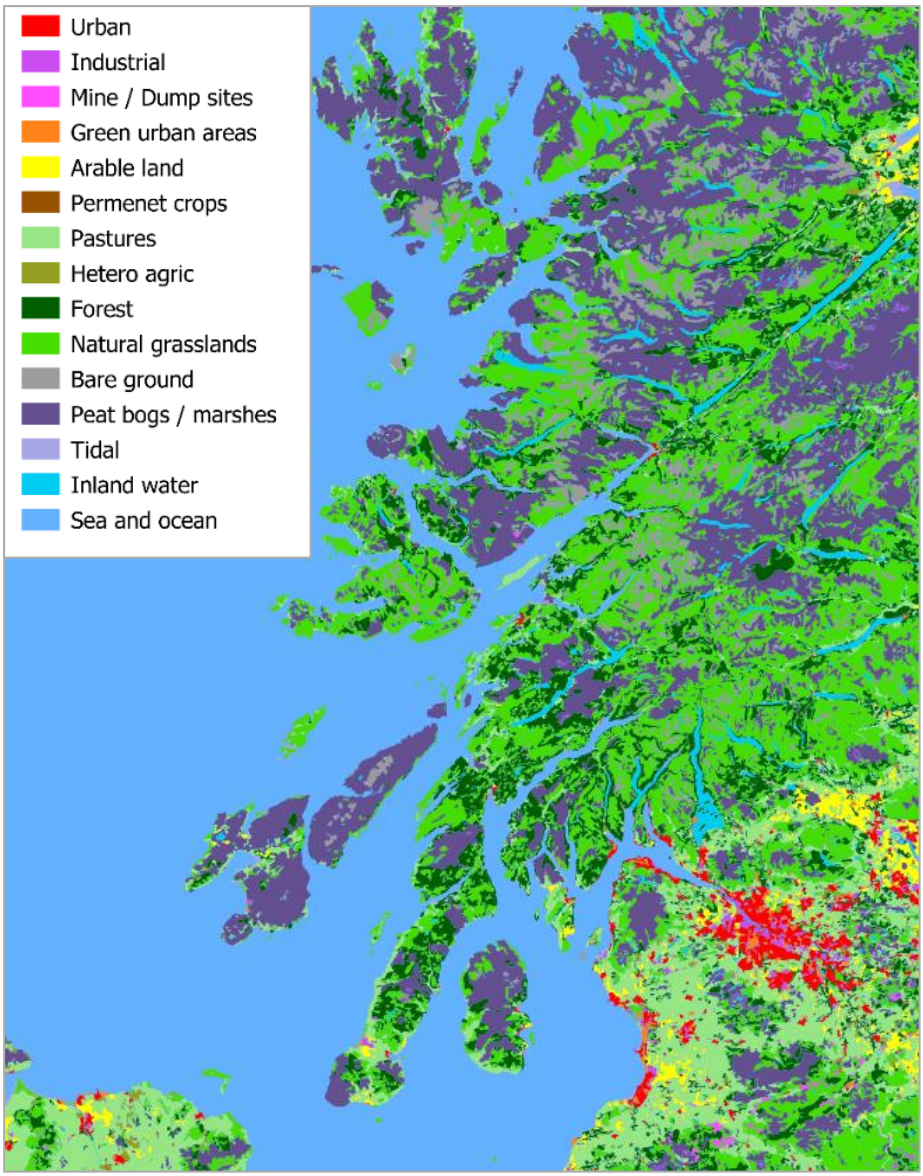

Figure 3.2. Land use in Argyll region of west Scotland. Based on EU CORINE land 2018 dataset. Much of the land in the island is dominated by either grassland or peat bog/marshes, while the mainland also includes forestry.

The aim of this study is to assess the population differentiation between four sites in Scotland (three islands and the mainland). This will then be placed in the wider context of the species by comparison to a population with a fully terrestrial distribution using the Irish populations which are, as discussed in Chapter 2, also considered stable but not separated by any significant bodies of water.

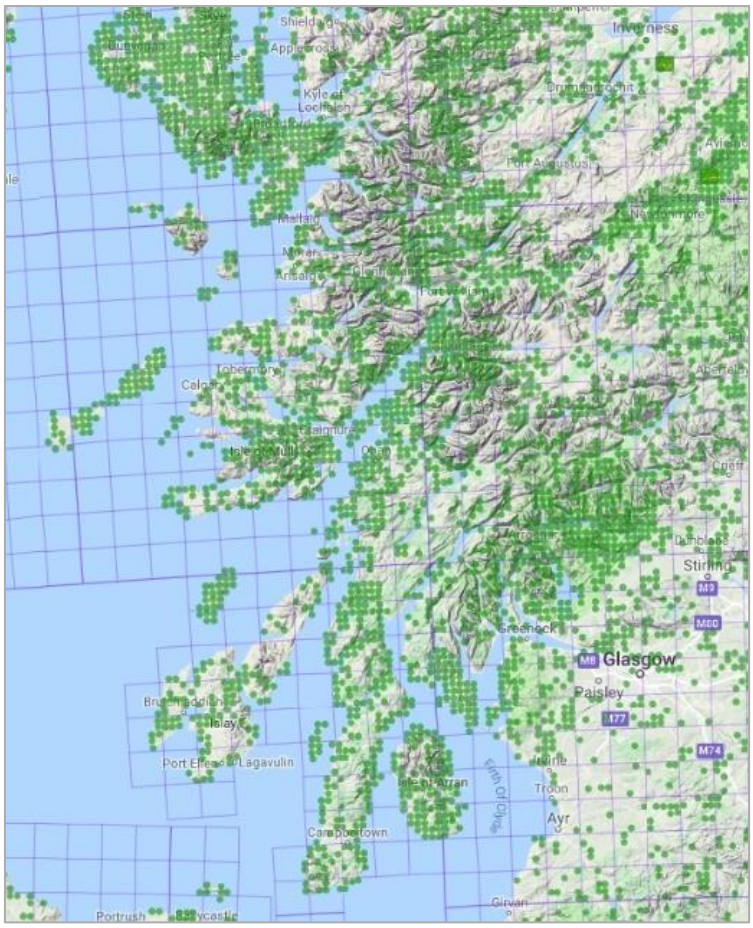

Figure 3.3. Occurrence of Devil's-bit scabious within 2km OS grid squares in the Argyll region. From BSBI (2019)

3.2 Method

3.2.1 Site selection and field sample collection.

Sites were selected based on prior knowledge of the presence of healthy (not declining or very small) populations of the Marsh Fritillary at the site. Selection was limited to those sites where safe access and sampling permission could be secured from the land owner or site manager. The legal requirement of a licence from Scottish Natural Heritage to collect Marsh Fritillary further limited site selection. Sites were selected to cover as many islands as was feasible and a mainland site for comparison. Straight line distances between populations was variable from 35.88 – 97.83km, minimum open water distances varied from 4.90 – 19.76km (Table 3.2).

Larvae were collected in autumn 2018 from populations on three islands (Mull, Islay and Oronsey) and one from a mainland site (Tayvallich) under Scottish Natural Heritage licence number 104772 (Table 3.1 & Figure 3.4). 222 additional larvae from Ireland were also included, as detailed in Chapter 2. Marsh Fritillary larvae are easily identified in the spring and autumn as they form sibling webs, all the offspring of a single female. A single larva per web was collected to prevent the collection of closely related individuals. A maximum of 30 larvae per site were collected as this was judged to give a fair representation of the genetic diversity at each site (Hale *et al.*, 2012; Smee, 2011).

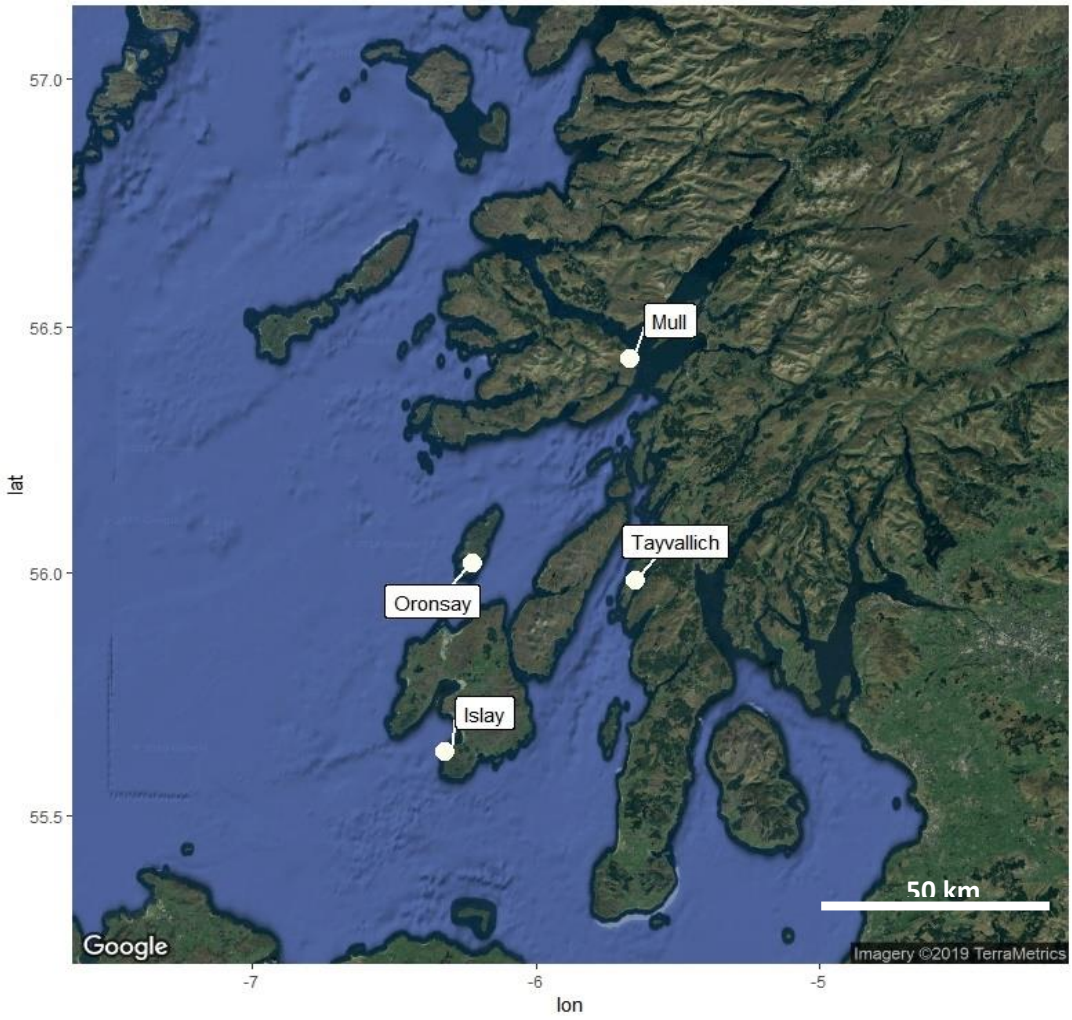

Figure 3.4. Three island sample sites (Mull, Oronsay and Islay) and one mainland site (Tayvallich) sampled for this study.

Table 3.1. Site information for sampling sites in Scotland. Regional climate information: Mean annual maximum temperature 12.2°C, mean annual minimum temperature 6.2°C, annual rainfall 1282.7mm (for detailed climatic data see Appendix C). Surrounding land use taken from EU CORINE land use data set, underlined land use is land use of site, other listed are land uses of surrounding area. Geological information

Site	Location	Elevation (m)	Site description	Surrounding land use	Bedrock geology	Sediment geology
Islay (island)	55.6325 / -6.3202	31.6	Rough grassland above coastal cliffs	Peat bogs	Alluvium - Clay, Silt, Sand and Gravel.	Till and Morainic Deposits (undifferentiated) - Diamicton, Sand and Gravel.
Mull (island)	56.4357 / -5.6691	8.3	Rough grassland between road and loch	Natural grasslands, Peat bogs, Pastures, Intertidal flats	Mull Lava Group - Basalt.	Raised Marine Beach Deposits of Holocene Age - Gravel, Sand and Silt.
Oronsay (island)	56.0207 / -6.2242	16.1	Rough grassland	Natural grasslands, Moors and heathland, Intertidal flats	Oronsay Greywacke Formation - Metamudstone.	Blown Sand - Sand.
Tayvallich (mainland)	55.9849 / -5.6493	4.7	Rough damp grassland bordered on two sides with woodland	Natural grasslands, Broad-leaved forest	Crinan Grit Formation - Quartzite.	Raised Marine Deposits - Gravel, Sand and Silt.

Table 3.2. Distances (km) between samples sites. Straight line distances below the line. Open water distances above line, the longest stretch of open water crossed on a minimum open water journey, allowing for the stepping stone effect of other major islands. Note that the open water distance given for Islay-Mull discounts the use of Colonsay/Oronsay and the mainland as stepping stones.

	Islay	**Mull**	**Oronsay**	**Tayvallich**
Islay	-	20	9	5
Mull	98	-	16	5
Oronsay	43	57	-	13
Tayvallich	57	50	36	-

3.2.2 Laboratory work and analysis

Specimens were stored at -80°C until used. Larval heads were used as the source and DNA extracted using the Livak method (Livak, 1984). Microsatellites previously developed for the Marsh Fritillary were used (Aurinia_01, Aurinia_16, Aurinia_45, Aurinia_70, (Smee *et al*., 2013) and Eau88 (Sinama *et al*., 2011)), these were amplified and scored according to the protocol in Appendix A. PCR products were separated via capillary electrophoreses using an AB3500 Genetic Analyser (Applied Biosystems) and sized relative to the LIZ500 (Applied Biosystems) internal size standard using GeneMapper 5. Sizes were checked manually and individuals with unclear peaks were reamplified and re-genotyped. Raw allele scores were binned using TANDEM to reduce error in the binning process.

Analysis was carried out in R (version 3.3.2) using binned allele sizes unless otherwise stated, no transformations of the data was carried out unless otherwise stated. To characterise the genetic variation within populations basic population statistics were calculated using diveRsity (Keenan et al., 2013). Pairwise Fst and Nei's G'st were calculated with corresponding p-values were calculated using strataG (Archer *et al.*, 2017) to determine the level of differentiation between populations. Spatial Principal Component Analysis (sPCA) were carried out, this was visualised as a 3-colour plot using package adegenet (Jombart, 2008). For details see Chapter 2 (section 2.2.2).

Isolation by distance (IBD) was tested using distance-based redundancy analysis (dbRDA (Meirmans, 2015)) this was carried out on a table of pairwise Fst values which was constrained by the geographical XY locations of the samples in order to calculate the proportion of the variation explained by location. This was done in vegan (Oksanen et al., 2019). Pairwise Fst and G'st values for the Scottish sites were compared to corresponding values for site pairs in Ireland of a similar separation distance (straight line distance +/- 10km the distance of any pair in Scotland). The significance of this was tested using a Walsh t-test, assuming unequal variance.

The statistical power of the microsatellites used was assessed in POWSIM 4.1 (Ryman & Palm, 2006), this used allele frequencies for the total dataset to carry out a Fisher's exact test with 10000 replicates.

3.3 Results

Power analysis showed that, based on detected allele frequencies and under a conservative estimate of an effective population size of 500 individuals (assuming all samples are unrelated and taking into account field observations), the microsatellites used would be able to detect Fst of 0.02 in 98.6% of cases and Fst of 0.05 in all cases which was deemed sufficient to answer the research question.

Three populations (Mull, Oronsey & Tayvallich) contained private alleles and showed significant deviations from HWE (Table 3.2). By contrast Islay had no private alleles and was at HWE. Fst and G'st showed significant population differentiation between all sites. Both Fst and G'st were highest between Islay and Tayvallich, while the second highest Fst was between Oronsay and Mull (0.1359) and the second highest G'st was between Oronsay and Islay (0.1296) (Table 3.3). Spatial Principal Component Analysis (sPCA) showed significant global structuring ($p<0.001$, $\lambda=0.181$)

but no significant local structuring (p>0.05, λ=0.091). There was no significant isolation by distance (p=0.542, F=0.689, df=2,1).

Table 3.3. Population genetics statistics for sites in Scotland. Total values are the totals across all sites except for allele richness which is a mean across all sites. Allelic richness is calculated using 1000 resamples (n=smallest input sample size), with replacement per population, and the mean value across all loci is give. Private alleles are given as the percentage of the total alleles across all loci found only in that population. H_e is average expected heterozygosity across all loci. H_o average observed heterozygosity across all loci. Fishers exact test with 1000 iterations was performed to detect significant departures from Hardy–Weinberg equilibrium (HWE).

Location	Sample size	Allelic richness	Private alleles (%)	H_e	H_o	HWE	F_{IS} (95% CI)
Islay	22	2.89	0	0.45	0.42	0.435	0.067 (-0.083, 0.208)
Mull	14	3.10	11.11	0.52	0.41	<0.01	0.215 (-0.022, 0.430)
Oronsay	30	3.65	16.00	0.56	0.41	<0.01	0.266 (0.116, 0.408)
Tayvallich	30	3.97	19.23	0.63	0.52	<0.001	0.181 (0.051, 0.310)
All Sites	96	3.40		0.65	0.46	<0.001	0.284 (0.218, 0.354)

Table 3.4. Pairwise Fst (below) and G'st (above) for all sites. Total Fst = 0.1239***, G'st = 0.1155***, n=96. Significance: * p<0.05; ** p<0.01; *** p<0.001.

	Islay (n=22)	Mull (n=14)	Oronsey (n=30)	Tayvallich (n=30)
Islay	-	0.0702**	0.1295***	0.1485***
Mull	0.1132**	-	0.1038**	0.0693***
Oronsey	0.1135***	0.1358**	-	0.1088***
Tayvallich	0.1485***	0.1030***	0.1132***	-

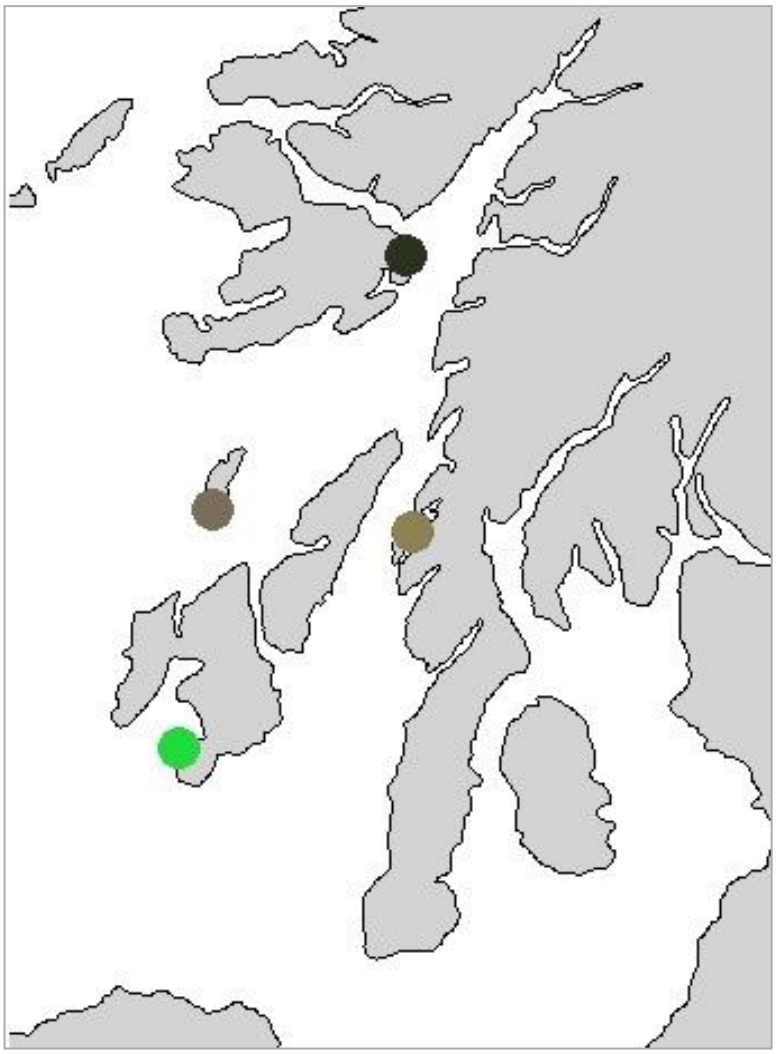

Figure 3.5. Colorplot for sPCA results for Scottish sites. The colours red, green and blue are assigned to the lagged scores of the first three principal components, respectively. Similarity of colour indicates genetic similarity

When compared with the pairwise Fst and G'st scores for Irish sites (Chapter 2, summary Table 3.5) it was found that the values obtained in Scotland were similar to those of Irish sites of a similar distance with the datapoints sitting within the range of the Irish datapoints (Figure 3.6). It should be noted that for the most part they cluster in the upper half of the range shown by the Irish samples (notably so for Fst and to a lesser extent G'st). When average pairwise Fst and G'st are compared for Scottish sites and Irish sites (with distances +/-10km of the Scottish distances), Scottish values are slightly higher than Irish (respectively; Fst=0.121 and 0.103,

G'st=0.105 and 0.097, n=222 and 94), however this difference was not found to be significant for either Fst (t=-1.70, df=18.63, p=0.11) or G'st (t=-0.52,df=9.94, p=0.62).

Table 3.5. Comparison of Scottish pairwise Fst and G'st values with Irish pairwise values where the distance is between sites is +/-10km of the distances in the Scottish dataset

Region	Fst			G'st		
	Min	Mean	Max	Min	Mean	Max
Scotland	0.1031	0.1213	0.1486	0.0694	0.1051	0.1486
Ireland	0.0293	0.1026	0.1671	0.0243	0.0971	0.1651

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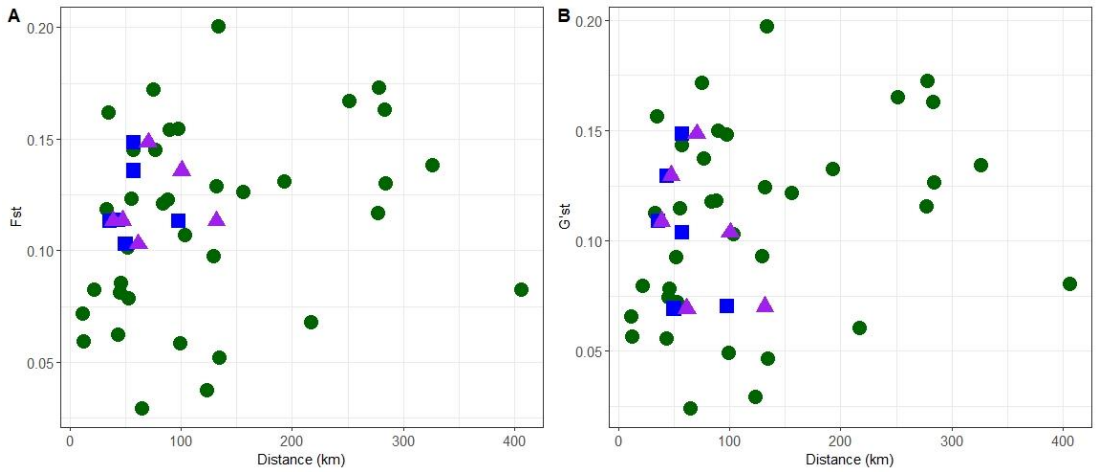

Figure 3.6. Pairwise Fst (A) and G'st (B) scores against straight line distance between sites. Irish datapoints (green) and Scottish datapoints (blue, direct line distance; purple, estimate of minimum journey distance incorporating minimum open water distance).

3.4 Discussion

There are significant levels of population differentiation between the various Scottish populations utilised in this study. Of the three island sites, Oronsey shows the highest allelic richness and number as well as the highest number of private alleles. This population is currently known to be expanding onto the nearby island of Colonsay (RSPB, pers. comm.) across a tidal causeway of approximately 90m at its narrowest point, though at low tide it is possible to walk between the islands. This high genetic

variation during a population expansion matches the high levels of phenotypic variation during a period of population expansion reported by Ford & Ford (1930).

This level of differentiation is comparable to that found between populations of the Meadow Brown butterfly (*Maniola jurtina*) on the Isles of Scilly (Baxter *et al*., 2017). The overall Fst for that study (0.0259, range 0.010-0.40) was lower than found in the current study (0.1239, range 0.1030-0.1485), however the Isles of Scilly are significantly smaller than the islands of the Inner Hebrides (the largest, St Mary's, is only slightly larger than Oronsey, the smallest included in this study, 6.58km^2 and 5.43km^2 respectively). The Isles of Scilly are also separated by less than 3km of open water, the shortest open water distance in this study is 4.90km (Tayvallich-Mull, requiring significant subsequent overland migration). Combining the geographic differences with the significantly higher movement index of 28 out of 45 (Dennis & Hardy, 2018) it is unsurprising that the population differentiation is less than has been found for the Marsh Fritillary in the Inner Hebrides. However, it must be noted that Baxter *et al*. (2017) did detect significant, though low, levels of population differentiation and therefore the detection of higher levels in the Inner Hebrides is consistent with previous findings for Lepidoptera in island settings.

There is no evidence of isolation by distance, in contrast to what was seen in the mainland population of Ireland. The lack of significant difference between pairwise Fst and G'st scores for comparable sites in Scotland and Ireland suggests that an island distribution presented similar barriers to dispersal as does the pastoral farming landscape of Ireland. The exact reason for this is unclear, however the minimum open water distance between sites are all within the maximum colonisation distance (15-20km) proposed by Warren (1994) and half are within 10km, a dispersal distance which has been recorded by Zimmermann *et al*. (2011). Movement between the sites of this study is possible while not encountering an open water distance in excess of 10km. This strongly suggests that the Marsh Fritillary may be using the islands of the

Inner Hebrides as stepping stones for dispersal, either as resting places during long distance movement or in a multigenerational fashion which would nevertheless result in gene flow between the islands and to or from the mainland.

It is possible that isolation by distance is only applicable above a certain distance and that below this landscape features have a greater impact on population differentiation. In Ireland there is a wide range of Fst/G'st values at distances <150km, above this distance the range of values is reduced. As the distances between sites in Scotland are <100km (straight line distance; sites <150km apart when taking into account minimum open water routes), it is possible that these sites are insufficiently separated for isolation by distance to be observable and other landscape features may be having a greater effect possibly resulting in isolation by environment (Wang & Bradburd, 2014). Isolation by environment has not been heavily studied in Lepidoptera however it has been detected, along with isolation by distance, in the peach fruit moth (*Carposina sasakii*) where landscape features such as topography form barriers to gene flow (Wang *et al.*, 2017).

Gillespie *et al*. (2012) proposed that highly dispersive taxa, which are primarily dispersed or assisted by wind, are more likely to arrive at a site in one long distance movement instead of multiple stepping-stone dispersal. By comparison islands as stepping stones have been suggested as a dispersal pattern for butterflies on the Torres Strait Islands between Papua New Guinea and Australia (Sands & New, 2008). Hence for poorly dispersive species, such as the Marsh Fritillary, the stepping-stone dispersal is more likely than a single long-distance movement.

It is also possible that the Marsh Fritillary displays multiple dispersal syndromes, that is patterns of covariance in morphology, behaviours and/or life history traits which are associated with dispersal (Ronce & Clobert, 2012). Where the ecology of patches varies, as it inevitably does, there is theoretically a balancing co-existence of two or more phenotypes (Doebeli & Ruxton, 1997) with an equilibrium

between the low and high dispersing forms (Mathias *et al.*, 2013). The spatial heterogeneity of an area can also favour one or the other phenotype (Hutson *et al*, 2001), though it has been suggested that some systems favour the evolution of an intermediary form rather than dimorphic phenotypes (Fronhofer *et al.*, 2011).

Evidence for these more and less dispersive phenotypes has been found in the Glanville Fritillary (*Melitaea cinxia*) (Hanski *et al.*, 2004), in this species it has also been determined that the dispersal rate is heritable mother to daughter though not mother to son (Saastamoinen, 2008). This is possibly due to the differing correlation between metabolic rate, as a proxy for flight capacity, and dispersal seen between the sexes, in females a high metabolic rate increases the likelihood of dispersal, while in males it increases territory patrolling (Niitepõld *et al.*, 2011). The heritability of dispersal tendency is explained at least in part by the variation in *Pgi* (phosphoglucose isomerase) genotype (Haag *et al.*, 2005). In the Glanville Fritillary a third of the variation in movement could be attributed to variations in the *Pgi* genotype with heterozygotes moving greater distances in cooler temperatures (Niitepõald *et al.*, 2009). Other studies have also detected an adaptive advantage for *Pgi* heterozygotes though the reason for this is still unclear, with overdominance (also called heterozygote advantage) and deleterious alleles at linked loci both proposed as explanations (Orsini *et al.*, 2009).

Although thus far most of the research on the effect of *Pgi* genotype on metabolism and dispersal in butterflies has been undertaken in the Glanville Fritillary, it is reasonable to assume, given the relatively close taxonomic relationship (both are members of the subfamily Melitaeinae (Barnard, 2011)), that similar processes may occur in the Marsh Fritillary, resulting in two or more dispersal syndromes. Variation in *Pgi* genotype has been detected in the Marsh Fritillary (Joyce & Pullin, 2001; Smee, 2011). Given what is known about the co-existence of multiple dispersal syndromes being at an equilibrium (Mathias *et al.*, 2013; Legrand *et al.*, 2015) which is affected

by environmental variables (Doebeli & Ruxton, 1997; Hanski *et al.*, 2006), it is also reasonable to assume that the equilibrium point is different in Scotland than in Ireland. This could contribute to the seeming lack of effect of island distribution on population differentiation.

The stepping-stone pattern of dispersal combined with occasional long-distance dispersals would function equally well in an island/mainland or strictly mainland system. It is also supported by the literature, with short and local dispersal being the most common dispersal type but longer distance dispersals occurring semi-frequently (Fric *et al.*, 2010; Junker & Schmitt, 2010; Zimmermann *et al.*, 2011; Konvicka *et al.*, 2012). In this way, the genetic differentiation of these island populations is less than might be expected and are at a level commensurate with the genetic differentiation of mainland populations.

There are two possibilities for island dispersal: short hops incorporating the shortest water distance with the remainder of the movement on land (probably being over multiple generations) or a single long-distance movement over water. Given what is known about typical dispersal distances in the Marsh Fritillary the former is more likely however the latter cannot be eliminated completely (Figure 3. 7).

The cumulative effect of the low pairwise Fst/G'st scores for adjacent populations (A->B->C->D->E) may result in a high pairwise Fst/G'st value if only the populations A and E are sampled. This can then be mistaken for the high values associated with occasional long-distance dispersal when what is actually occurring is frequent short distance dispersal between stepping-stone populations over multiple generations. To confirm if this is occurring in the Southern Inner Hebrides, it would be recommended to undertake further sampling of populations with the potential to function as stepping-stones. These would be along the mainland coastal region between Oban (the mainland closest to Mull) and Tayvallich and the east coast of Jura (stepping-stone sites for dispersal between Tayvallich and Islay). Landscape

genetic modelling would also be beneficial to assess the exact impact of water as a barrier to dispersal.

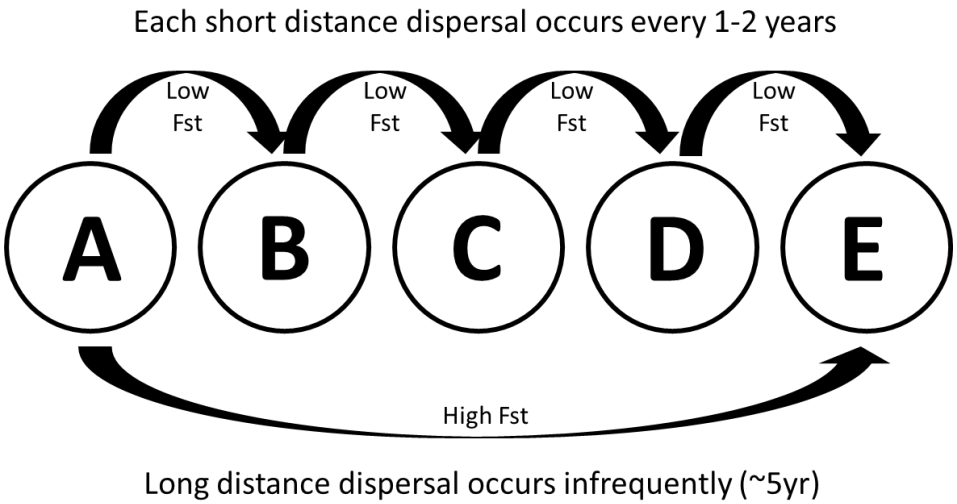

Figure 3.7. Two possible dispersal routes between populations A and E, assuming dispersals over short distances occur with greater frequency than those over long distances. The multi-generational stepping-stone model incorporates populations B, C and D. A single long-distance dispersal directly from A to E is also possible.

4 Celtic connections? Utilising the potential of genetic information for conservation management.

4.1 Introduction

There are only finite resources available for conservation (Leader-Williams & Albon, 1988). Therefore, the question arises as to how these resources should be best utilised, given that inappropriate application of resources can potentially lead to the failure to conserve a particular species (Wilson *et al.*, 2006; Restani & Marzluff, 2006; Murdoch *et al*., 2007; Bottrill *et al.*, 2008; Joseph *et al.*, 2009). One of the questions that must be addressed is at which taxonomic level should the protection focus.

At an international level it is the taxonomic level of species which is afforded threatened status (IUCN, 2019) and protection (for example the EU Habitats Directive 92/43/EEC and later amendments). However at national and regional levels the conservation of subspecies and specific populations becomes a concern (Gippoliti & Amori, 2007; Casacci *et al*., 2014). The independent conservation of subspecies and other specific populations, sometimes called evolutionarily significant units (ESUs), is of additional concern when captive breeding is an appropriate conservation strategy. (Ryder, 1986; Maguire & Lacy, 1990; Michaux *et al*., 2004).

For the purpose of this study the term ‘population’ will be used in the metapopulation sense, a group- of individuals with significantly higher intra-group interactions than inter-group interactions (*sensu* Hanski (1997)). ‘Patch’ is used to define a group within a patchy population where intra-group and inter-group interactions occur at a similar frequency (*sensu* Hanski & Thomas (1994)). ‘Colony’ is used to define samples collected from close geographical proximity without implication to the population structuring. ‘Area’ refers to a group of colonies or samples in geographical proximity.

For a species with a metapopulation structure at least some effort must be made to conserve the individual populations (McDonald-Madden *et al*., 2008). However, the exact definition of a population, and indeed a metapopulation, has never been defined explicitly (Millstein, 2010). Many species will have a non-continuous distribution but if the level of interactions between patches is not significantly less than within patches then it is not a true metapopulation and would be more accurately described as a patchy population (Millstein, 2010).

Two types of metapopulation are generally recognised; classical (also called Levins) and source-sink (also called mainland-island), although these may be viewed as two ends of a spectrum (Figure 4.1). Patchy populations are often included in discussions of metapopulation dynamics but are not strictly metapopulations due to the high levels of inter patch exchange described above (Hanski & Thomas, 1994; Harrison & Taylor, 1997).

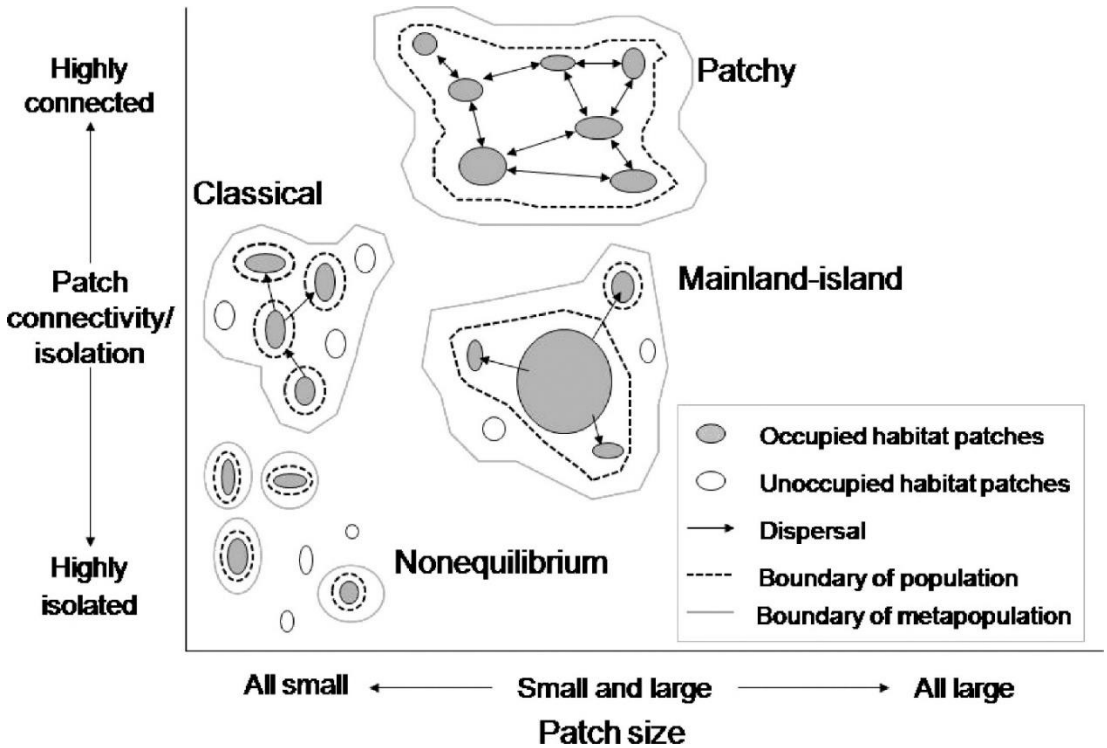

Figure 4.1. Types of metapopulations, their patch size and connectivity. From Aycrigg & Garton (2014)

Different metapopulation types have different patterns of genetic differentiation between colonies and these can be used to assess the type of metapopulation that the colony belongs to (Aycrigg & Garton, 2014). Typically, classical metapopulations have moderate patch differentiation compared to patchy populations which have very low-level differentiation while nonequilibrium structures have much higher levels. Source-sink dynamics usually have genetic differentiation levels between classical and patchy (Table 4.1). These may be reflected in different levels of pairwise Fst values (Aycrigg & Garton, 2014). However, these are likely to be species specific and no broad values could be found in the literature.

Determining the nature of a patch occupied by an endangered species (usually termed a colony in butterfly ecology) is critical for implementing appropriate conservation measures (Table 4.1). However there is often a disconnect between the theory and the practice (Pullin *et al*., 2004). This is often due to factors such as the limited time that conservation managers have to read scientific literature (Fabian *et al*., 2019) and problems with accessing that literature due to delays in publication or paywalls (Cvitanovic *et al.*, 2014; Fuller *et al.*, 2014). For this reason, metapopulation theory may be incorrectly applied and each colony managed as an independent population. When this occurs, it can result in a sub-optimal use of the limited resources available for conservation work.

Identifying suitable targets for conservation is important. When species exist across a broad landscape, knowing which areas to target as a conservation priority is vital. Although some benefit may be derived from targeting the largest populations it is also important to consider the need to conserve the maximum amount of genetic diversity. Landscape genetics combines the fields of population genetics with landscape ecology (Manel *et al*., 2003), and provides a theoretical and technical framework to identify units for conservation priority due to unique patterns of genetic

Table 4.1. Characteristic and conservation priority for individual patches within differing metapopulation types, including patchy and non-equilibrium.

Metapopulation type	Patch genetic differentiation (Fst / G`st)	Patch size	Patch connectivity	Patch extinction probability	Conservation priority for individual patch
Classical	Moderate	All similar, usually relatively small	Low-moderate, bidirectional	Moderate	Moderate. Natural recolonisation is possible with time but disruption to wider dynamic is a risk
Source-Sink	Low to moderate	Variable. Large (source) - Small (sink)	Low-moderate, unidirectional	High (sink), Low-none (source).	High (source) - Low (sink). Loss of the source habitat will disrupt the dynamic, sink habitat a lower priority as recolonisation likely unless habitat is totally removed
Patchy	Low	Small	High, bidirectional	Low	Low. Natural recolonisation likely within a short period if habitat is not totally removed.
Non-equilibrium	High	Usually small	None	High	High. Natural recolonisation is not possible, wider conservation efforts at the landscape scale may restore functioning dynamic

diversity as well as identify landscape barriers, natural or anthropogenic, to gene flow (Manel & Holderegger, 2013). This can then be used to identify suitable management units and inform management plans and interventions.

Landscape features have been shown to isolate populations and reduce gene flow in Lepidoptera (Keyghobadi *et al.*, 1999) and that reduced connectivity results in increased Fst (Keyghobadi *et al.*, 2005). Landscape scale population differentiation has been detected in the checkerspot butterfly *Euphydryas editha,* including varying patterns of isolation across different parts of its range (Baughman *et al*., 1989). These patterns were suggested to be the result of the interaction between drift, selection, migration and historic events though the specific details of these interactions were not confirmed. Landscape features have also been shown to affect isolation in the Bog Fritillary (*Boloria eunomia*) within a relatively small ($150m^2$) study area. This effect of landscape in isolating populations occurs in part due to surrounding habitat type. The Meadow Brown (*Maniola jurtina*) has been shown to disperse more frequently through landscape areas which are more similar to its habitat type (Villemey *et al.*, 2016). Therefore, less similar habitat can act as a barrier to dispersal and serve to isolate a population.

Landscape ecology and metapopulation theory work on two different perspectives but are highly complementary to each other when informing the conservation management decisions for an endangered species. At a broad scale, landscape ecology can identify areas which should be a conservation priority due to their unique genetic variation (Allendorf *et al.*, 2010; Funk *et al.*, 2012). Once such an area has been identified, metapopulation theory can then be applied to determine which patches are part of a patchy population and which may be functioning as independent populations within the metapopulation. Reed buntings (*Emberiza schoeniclus*) live in discreet areas of reeds and it was believed that each area was a population within a metapopulation, however genetic analysis revealed that there was

sufficiently high gene flow between the areas that the population dynamic was that of a patchy population rather than a metapopulation (Mayer *et al*., 2009).

The Marsh Fritillary butterfly (*Euphydryas aurinia*) is declining throughout its range and many places within the British Isles have seen a reduction in abundance or area of occurrence within the last 50 years, including regional extinctions. It is protected under the Wildlife and Countryside Act 1981 and the subject of a number of conservation interventions in various regions (Butterfly Conservation, 2018a). The Marsh Fritillary has a classic metapopulation structure (Warren, 1994), colonies of the Marsh Fritillary being found within patches of the larval host plant, Devil's-bit scabious.

It is important to understand how geographical distances impact population differentiation, to provide a better understanding of what constitutes a patch within a population and a population within a metapopulation.

The majority of Marsh Fritillary populations in England are now found in the south west. There are considered to be only a dozen colonies in Cornwall with several large populations, notably around Bodmin Moor, and several small populations with less than 100 adults per year (Curtis & Maclean, 2016). The county has also experienced recent population declines at previously strong sites such as Goss Moor and regional extinction is suspected in West Penrith (Curtis & Maclean, 2016).

The Lizard peninsula in Cornwall is the most southerly point in Britain. Only a handful of Marsh Fritillary colonies are known from the Lizard and several are in close geographical proximity. Presently all known colonies are considered and managed as independent populations within a metapopulation (Curtis, pers. comm.). However, no genetic study has previous been undertaken to determine the level of differentiation between the sites. Intensive sampling can clarify whether what is considered a population from a management perspective is indeed a biological population *sensu* Ruggiero *et al*. (1994).

In Wales the Marsh Fritillary was once widely distributed but has suffered declines during the second half of the twentieth century (Figure 4.2). Nevertheless it has remained relatively strong and between 1990-2005 over 200 colonies were identified (Fowles & Smith, 2006). Warren (1994) reported that the largest known population in the UK at the time was from Rhos Llawr-Cwrt in west Wales, a 40ha site believed to contain 30,000 adults. Despite this abundance at some sites, throughout the 1990s and early-2000s the general trend of the Marsh Fritillary in Wales was a decline, though with notable years of high abundance such as 1998. From 2006 this decline was reversed, reaching a peak around 2014 before plateauing, followed by a minor decline in recent years (Butterfly Conservation, 2019).

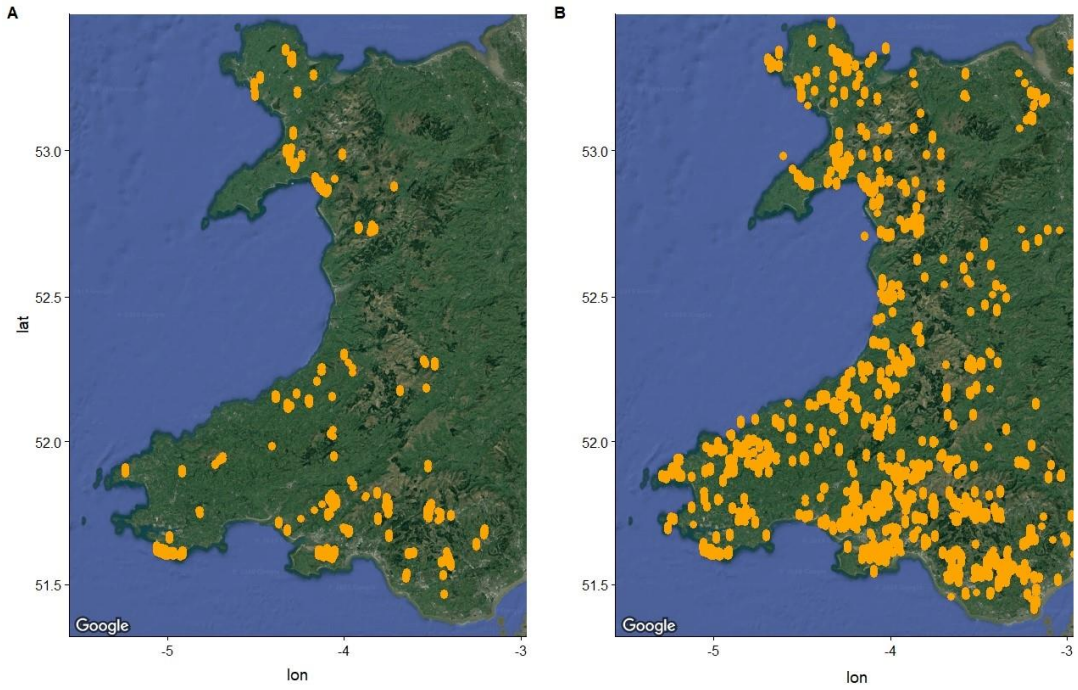

Figure 4.2. Current (A) and pre-1900 (B) distributions of the Marsh Fritillary in Wales. The populations have been separated into north and south populations. This study will focus on part of the southern population

The majority of the land in Wales continues to be either natural grassland or pastoral farmland (EEA, 2018), in notable contrast to the nearby regions of England which are predominantly arable farmland or urban development (Figure 4.3). Poor

habitat in Wales may have contributed to the decline of the Marsh Fritillary. A survey of the available grassland, based on data collected 2000-2005, determined that of the 15.9% of grassland in Wales capable of supporting the Marsh Fritillary, only 11.85% of the habitat surveyed was of “Good Condition”, with the rest suffering from either inappropriate management or a lack of management (Fowles & Smith, 2006). Since this study the implementation of appropriate management practices including a landscape level approach have resulted in an increase in numbers of individuals and known populations, although it has not returned to the level recorded in 1993 (Butterfly Conservation, 2019).

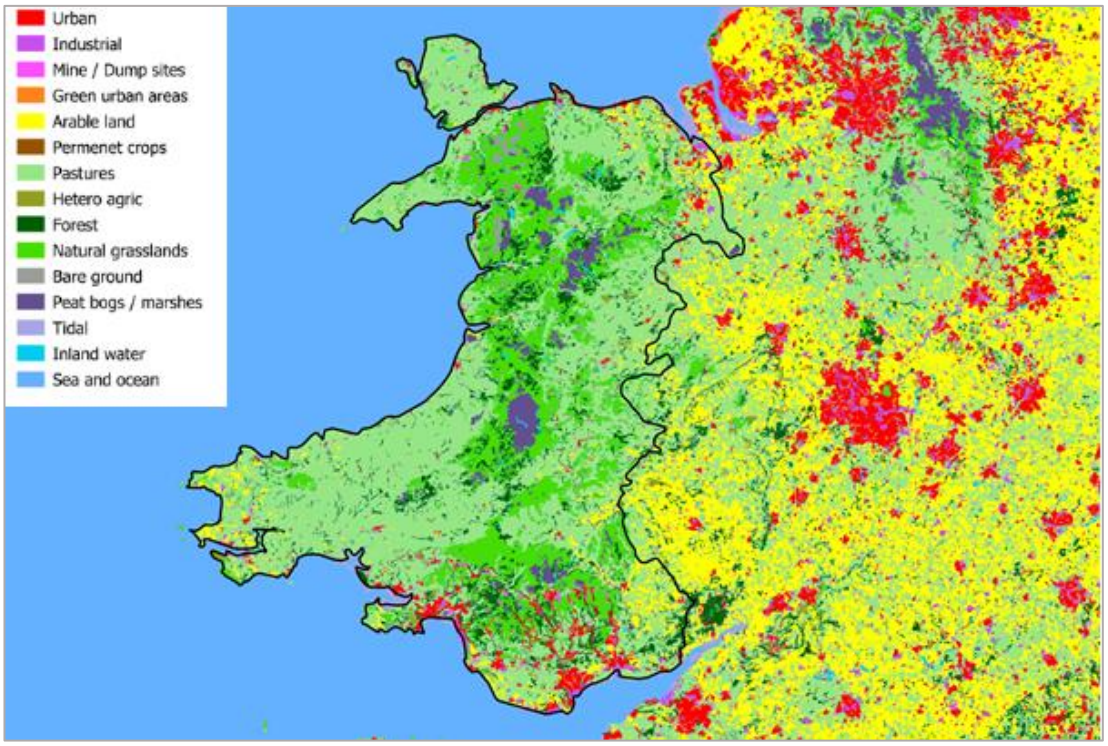

Figure 4.3. Land use in Wales (outlined region) is predominantly pastoral with some limited areas of arable farming and urban development, notably on the south coast. This is in contrast to the nearby regions of England which show extensive areas of both arable farming and urban development.

Previous assessments of the dispersal ability of the Marsh Fritillary have produced variable results (Table 1.1). While most studies agree that dispersals over 2km occur, Junker & Schmitt (2010) recorded a mean of under 100m, with a few individuals moving greater than 300m. Fric *et al*. (2010) and Konvicka *et al*. (2012)

both report dispersal over distances of 2-3km while Zimmermann *et al*., (2011) and Warren (1994) found dispersal distances of 10km and 15-20km respectively. These studies used Mark Release Recapture techniques to assess dispersal ability, gene flow patterns can also be used to assess dispersal and have suggested dispersal distances of up to 10-14km for the Marsh Fritillary (Sigaard *et al*., 2008). What has thus far not been addressed in the literature is the geographical distance which differentiates patches (where inter-area movement is similar to intra-area movement) and populations (where inter-area movement is significantly lower than intra-area movement).

Landscape genetics is the combination of molecular genetics and landscape ecology, it involves the spatial mapping of allele frequencies and the correlation of these with the current landscape (Manel *et al.*, 2003) and sometimes the historic landscape (Orsini *et al.*, 2008). It can be used to assess genetic structure and functional connectivity (that is, the degree to which the landscape facilitates or impedes dispersal) (Manel & Holderegger, 2013; Waits *et al.*, 2016). These genetic patterns depend on temporal and spatial scale, this can be used to assess population dynamics and the impact of conservation measures to increase connectivity (Waits *et al.*, 2016). Landscape patterns of genetic diversity can also be used to determine management units which are a conservation priority in order to conserve the maximum level of genetic diversity (Manel & Holderegger, 2013).

In Lepidoptera, landscape genetics has commonly been used to assess the effect of fragmentation on genetic structuring. Changes in land use have been shown to reduce the connectivity between populations of Violet Copper butterflies (*Lycaena helle*) and increase the level of differentiation between populations (Habel *et al.*, 2011). In the Meadow Brown butterfly (*Maniola jurtina*), it has been shown that dispersal (as measured by gene flow) is more common through landscape elements which are most similar to suitable habitat (Villemey *et al.*, 2016). For Hesperid

butterflies of the *Thymelicus* genus it has been demonstrated that landscape has different impacts on gene flow in closely related species and that recent changes in gene flow patterns have resulted from habitat alterations (Engler *et al.*, 2014). However this is not true for all species; population demographics have a greater impact on genetic structuring than landscape features in the alpine butterfly *Colias behrii* (Schoville *et al.*, 2012). Landscape genetics has also been used to examine the effect of management techniques on pest species, the genetic structure of the Coddling moth (*Cydia pomonella*) was shown not to be affected by the management status (in-production or abandoned) of the orchards where it is a pest (Fuentes-Contreras *et al.*, 2015). Thus far there have been no landscape genetics studies published in the Marsh Fritillary.

A previous unanalysed data set exists for the Marsh Fritillary in South Wales. Instead of intensively sampling a limited number of geographical locations, this data set is the result of low population sampling size but extended across a broad geographical area. This allows broad patterns of genetic variation to be assessed and possible management units and priorities to be identified.

This chapter aims to use patterns of genetic variation to inform management practices. First at a local level it will assess if there is a genetic basis for the decision to conserve all patches of Marsh Fritillary on the Lizard Peninsula as independent populations. Second it will assess the distribution of genetic variation across a broad area of South Wales and identify possible management units and conservation priorities.

4.2 Method

4.2.1 Site selection and field sample collection.

Genetic investigation of the Lizard population was invited by Dr Robin Curtis (University of Exeter) who is involved in the conservation of the Marsh Fritillary in the area. Sites were selected under his direction to answer the question of the suitability of the current management practices, taking into account which sites had sufficiently large populations, could be safely accessed and licensing considerations. The sites chosen were two pairs of sites, currently managed as four separate populations. Within pair site separation was within the widely accepted distance of the Marsh Fritillary (<400m) while the separation between the pairs exceeded some reported dispersal distances (>3km) (Table 4.2).

Larvae were collected in Spring 2018 (under Natural England licence number 2016-25165-SCI-SCI) (Figure 4.4, Table 4.3). A single larva per web was collected to prevent the collection of closely related individuals. A maximum of 30 larvae per site were collected as this was judged to give a fair representation of the genetic diversity at each site (Hale *et al*., 2012; Smee, 2011).

Data for South Wales was supplied by Jon Hudson (University of Birmingham) and collected under licence from Countryside Council for Wales (now National Resources Wales). These larvae were collected from 33 geographic locations in 2011-2012, these were aggregated into eleven populations based on sampling information supplied and geographical proximity (Figure 4.5). DNA extraction, microsatellite amplification and scoring were carried out by Nevada Genomics, using 14 microsatellites published by Petenian *et al*. (2005) and Sinama *et al*. (2011) (Appendix D).

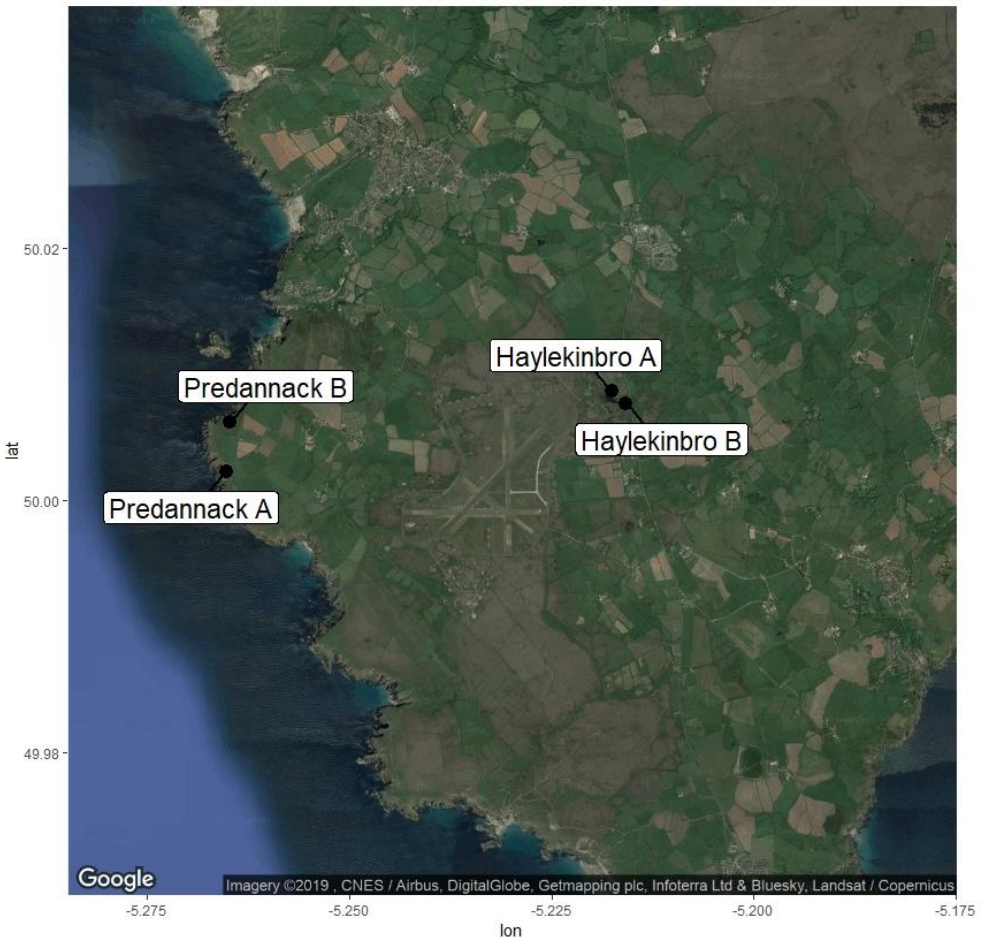

Figure 4.4. Sites on the Lizard Peninsula in Cornwall are divided into two areas Predannack and Haylekimbro. Each area is then subdivided into two sites which are currently managed as independent populations.

Table 4.2. Distance between sites in Cornwall (km), average distance between Predannack and Haylekimbro is 3.49km.

	Predannack B	Haylekimbro A	Haylekimbro B
Predannack A	0.441		
Predannack B	3.476	3.386	
Haylekimbro A	3.580	3.505	0.165

Table 4.3. Site information for sampling sites on the Lizard Peninsula, Cornwall. Regional climate information: Mean annual maximum temperature 13.7°C, mean annual minimum temperature 7.9°C, annual rainfall 1607.8mm (for detailed climatic data see Appendix C)Surrounding land use taken from EU CORINE land use data set, underlined land use is land use of site, other listed are land uses of surrounding area. Geological information form British Geological Society.

Site	Location	Elevation (m)	Site description	Surrounding land use	Bedrock geology	Sediment geology
Predannack A	50.002265 / -5.2651835	61.6	Grassland above costal cliffs enclosed by electric fence to deter sheep	Pastures, Non-irrigated arable land	Traboe Hornblende-schist (lizard Complex) - Schist, Hornblende.	Alluvium - Clay, Silt, Sand and Gravel.
Predannack B	50.006225 / -5.2648099	47.4	Grassland above costal cliffs partly enclosed by electric fence to deter sheep	Pastures, Non-irrigated arable land	Traboe Hornblende-schist (lizard Complex) - Schist, Hornblende.	Alluvium - Clay, Silt, Sand and Gravel.
Haylekimbro A	50.00867 / -5.2175836	86.2	Rough damp grassland (near small lake), hedges including some trees	Moors and heathland, Pastures, Natural grasslands, Land principally occupied by agriculture, with significant areas of natural vegetation	Lizard Complex - Peridotite and Serpentinite.	Alluvium - Clay, Silt, Sand and Gravel.
Haylekimbro B	50.00772 / -5.2158171	85.1	Rough damp grassland (near small lake), hedges including some trees	Moors and heathland, Pastures, Natural grasslands, Land principally occupied by agriculture, with significant areas of natural vegetation	Lizard Complex - Peridotite and Serpentinite.	Alluvium - Clay, Silt, Sand and Gravel.

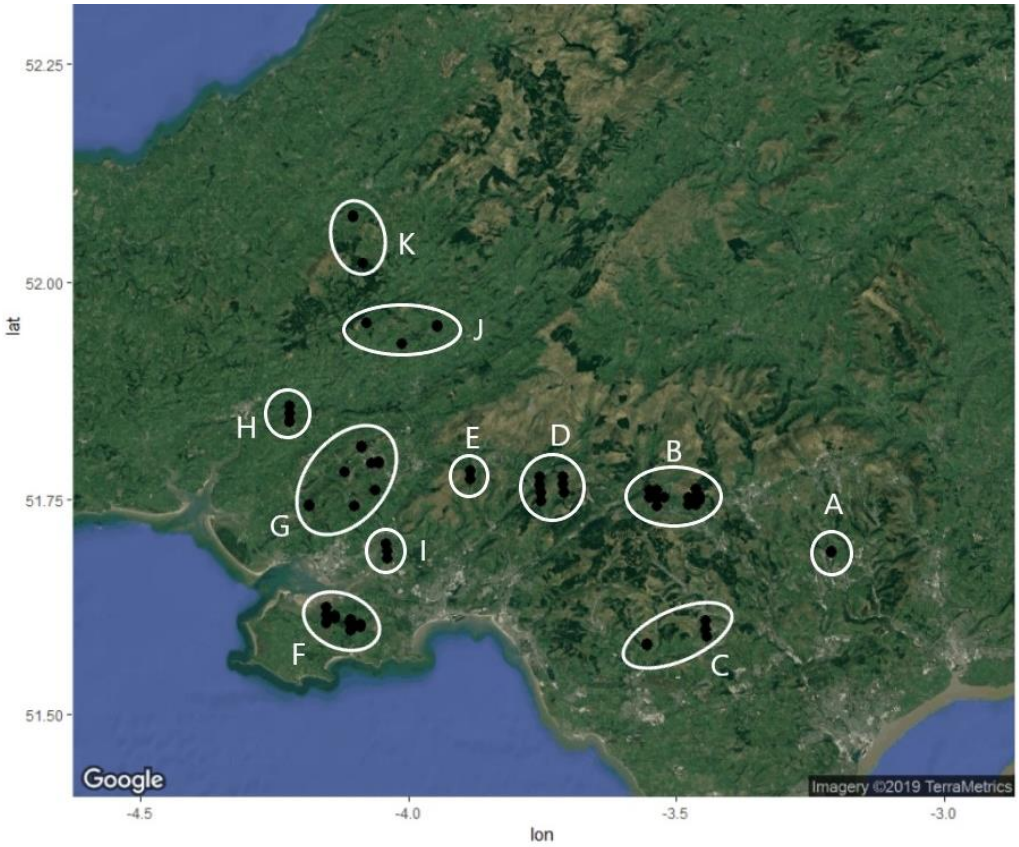

Figure 4.5. Sample sites in south Wales, divided into 11 areas (A-K) based on sampling information provided and geographical proximity. Regional climate information: Mean annual maximum temperature 13.5°C, mean annual minimum temperature 8.5°C, annual rainfall 999.2mm for detailed climatic data see Appendix C).

4.2.2 Laboratory work and analysis

Specimens were stored at -80°C until used. Larval heads were used as the source and DNA extracted using the Livak method (Livak, 1984). Microsatellites developed by Smee *et al*. (2013) were used (Aurinia_01, Aurinia_13, Aurinia_16 & Aurinia_64), these were amplified using the protocol in Appendix A. PCR products were separated via capillary electrophoreses using an AB3500 Genetic Analyser (Applied Biosystems) and sized relative to the LIZ500 (Applied Biosystems) internal size standard using GeneMapper 5. Sizes were checked manually and individuals with unclear peaks were reamplified and re-genotyped. Raw allele scores were binned using TANDEM to reduce error in the binning process. Raw allele scores for the Welsh

dataset were also binned using TANDEM, independently of the samples from Cornwall.

Analysis was carried out in R (version 3.3.2) using binned allele sizes unless otherwise stated, no transformations of the data was carried out unless otherwise stated. To characterise the genetic variation within populations basic population statistics were calculated using diveRsity (Keenan et al., 2013). Pairwise Fst and Nei's G'st were calculated with corresponding p-values were calculated using strataG (Archer *et al.*, 2017) to determine the level of differentiation between populations. Isolation by distance (IBD) was tested using distance-based redundancy analysis (dbRDA (Meirmans, 2015)) this was carried out on a table of pairwise Fst values which was constrained by the geographical XY locations of the samples in order to calculate the proportion of the variation explained by location. This was done in vegan (Oksanen et al., 2019). Spatial Principal Component Analysis (sPCA) were carried out, this was visualised as a 3-colour plot using package adegenet (Jombart, 2008). For full detail see Chapter 2 (section 2.2.2). Landscape resistance analysis was used to test effect of elevation on gene flow patterns; a resistance matrix was constructed based on relative elevations using Circuitscape (Shah & Mcrae, 2008), this was then tested against the pairwise Fst and G'st scores using multiple regression on distance matrices in `ecodist` (Goslee & Urban, 2007)

The statistical power of the microsatellites used was assessed in POWSIM 4.1 (Ryman & Palm, 2006), this used allele frequencies for the dataset to carry out a Fisher's exact test with 10000 replicates. Datasets for Cornwall and Wales were tested separately.

4.3 Results

4.3.1 Cornwall

Power analysis showed that, based on detected allele frequencies and under a conservative estimate of an effective population size of 500 individuals (assuming all samples are unrelated and taking into account field observations), the microsatellites used would be able to detect Fst of 0.02 in 94% of cases and Fst of 0.05 in all cases which was deemed sufficient to answer the research question.

Pairwise Fst and G'st between sites in the same area were found to be low and non-significant in all cases (Table 4.4), however pairwise values for both Fst and G'st were significant for Haylekimbro A – Predannack A and Haylekimbro A – Predannack B. When both sites in an area are amalgamated for analysis, the two areas have identical allele number, richness and percentage private alleles (Table 4.5). Both areas also showed deficiency in the number of observed heterozygotes and significant departures from HWE

Table 4.4. Pairwise Fst (below line) and G'st (above line), for all sites in Cornwall. Significant scores are denoted as follows: * p<0.05; ** p<0.01; *** p<0.001. Pairwise Fst and G'st between the two areas are 0.070 and 0.0597, respectively, both p<0.0010, $n_{Pendannack}$=52, $n_{Haylekimbro}$=32.

	Predannack A (n=30)	**Predannack B (n=22)**	**Haylekimbro A (n=29)**	**Haylekimbro B (n=3)**
Predannack A	-	-0.0013	0.0369**	-0.0405
Predannack B	0.0149	-	0.0817***	0.0885
Haylekimbro A	0.0507**	0.0961***	-	-0.182
Haylekimbro B	0.0393	0.0719	-0.0669	-

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Table 4.5 Population genetics statistics for sites in Cornwall. Total values are the totals across all sites except for allele richness which is a mean across all sites. Allelic richness is calculated using 1000 resamples (n=smallest input sample size), with replacement per population, and the mean value across all loci is give. Private alleles are given as the percentage of the total alleles across all loci found only in that population. H_e is average expected heterozygosity across all loci. H_o average observed heterozygosity across all loci. Fishers exact test with 1000 iterations was performed to detect significant departures from Hardy–Weinberg equilibrium (HWE).

Site	**Sample size**	**Allelic richness**	**Private alleles (%)**	**Ho**	**He**	**HWE**	**Fis (95% CI)**
Predannack A	30	2.18	11.11	0.43	0.47	<0.001	0.083 (-0.054 – 0.223)
Predannack B	22	1.88	0	0.28	0.4	<0.001	0.285 (0.030 – 0.516)
Haylekimbro A	29	2.23	4.76	0.38	0.49	<0.001	0.285 (0.030 – 0.356)
Haylekimbro B	3	2.04	0	0.4	0.38	0.322	-0.059 (-0.737 – 0.204)
Predannack	52	3.69	9.52	0.37	0.45	<0.001	0.174 (0.040 – 0.314)
Haylekimbro	32	3.93	9.52	0.39	0.49	<0.001	0.206 (0.040 – 0.365)
Total	84	4.41		0.38	0.48	<0.001	0.22 (0.126 – 0.315)

4.3.2 South Wales

Power analysis showed that, based on detected allele frequencies and under a conservative estimate of an effective population size of 1000 individuals (assuming all samples are unrelated), the microsatellites used would be able to detect Fst of 0.01 in 75% of cases and Fst of 0.02 in all cases which was deemed sufficient to answer the research question.

Sample size varies markedly between populations (n=2-18, $\bar{x}$=7.55, SD= 5.61 Table 4.4). Per area allelic richness is similar across all areas. All areas contain private alleles and exhibit heterozygote deficiency. Most areas show significant departures from HWE except for areas A, I and K.

Spatial principal component analysis (sPCA), revealed significant global structuring ($p<0.001$, $\lambda=1.562$, $n=83$) but no significant local structuring ($p>0.05$, $\lambda=0.898$). Some areas are more similar than others with the eastern region showing a greater level of diversity compared to the west (Figure 4.6).

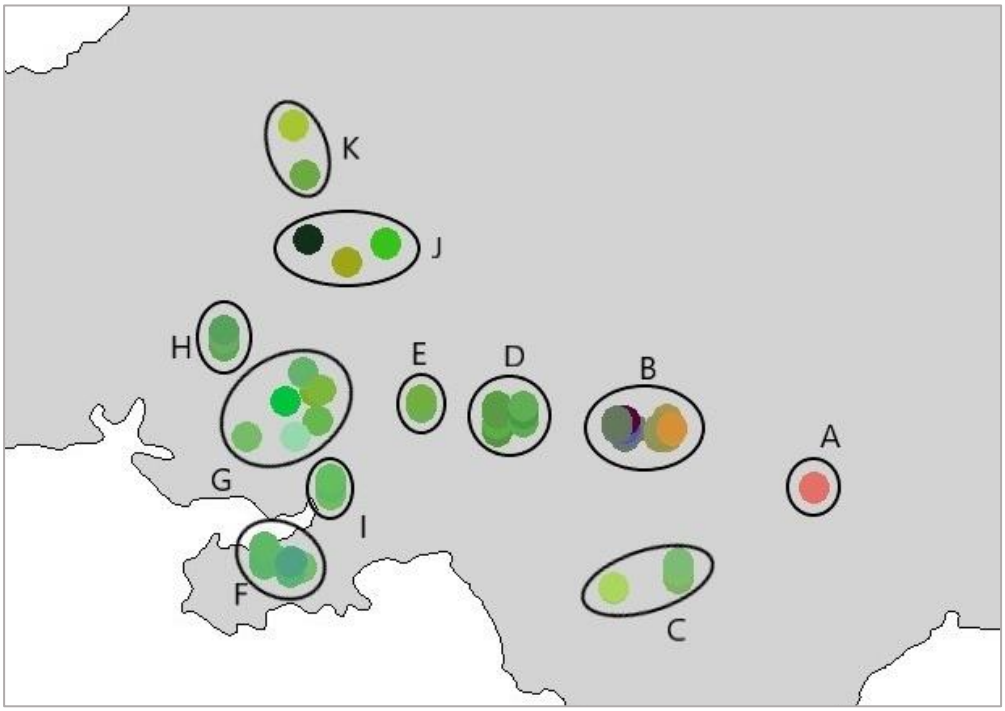

Figure 4.6. Colorplot of sPCA for South Wales showing areas A-K, there is significant global structuring ($p<0.001$).

ble 4.6. Population genetics statistics for sites in South Wales. Total values are the totals across all sites except for allele richness which is a mean across all sites. Allelic richness is calculated using 1000 resamples (n=smallest input sample size), with replacement per population, and the mean value across all loci is give. Private alleles are given as the percentage of the total alleles across all loci found only in that population. H_e is average expected heterozygosity across all loci. H_o average observed heterozygosity across all loci. Fishers exact test with 1000 iterations was performed to detect significant departures from Hardy–Weinberg equilibrium (HWE).

Area	Sample size	Allelic richness	Private alleles (%)	Ho	He	HWE	Fis (95% CI)
A	3	1.66	12.9	0.24	0.43	1	0.444 (-0.703, 0.742)
B	18	1.94	6.55	0.27	0.6	<0.001	0.542 (0.436, 0.640)
C	6	1.8	4.54	0.26	0.47	<0.001	0.440 (0.197, 0.573)
D	7	1.88	2.04	0.27	0.54	<0.001	0.513 (0.371, 0.579)
E	2	1.56	15.38	0.25	0.31	<0.01	0.200 (-0.388, 0.812)
F	17	1.92	14.28	0.28	0.57	<0.001	0.510 (0.407, 0.606)
G	13	1.88	6.45	0.25	0.56	<0.001	0.559 (0.439, 0.674)
H	8	1.95	3.5	0.29	0.58	<0.001	0.551 (0.266, 0.702)
I	3	1.58	3.57	0.21	0.38	1	0.443 (-0.701, 0.991)
J	2	1.72	3.44	0.36	0.39	<0.05	0.091 (-0.452, 0.639)
K	4	1.77	2.63	0.25	0.49	0.09	0.489 (0.047, 0.650)
Total	83	6.61		0.27	0.62	<0.001	0.571 (0.526, 0.612)

Many of the samples from the west are of similar colouring, indicating genetic similarity. In the east however there are two areas, A and B, which are notably different to the western areas as well as to each other. These two areas also contain the majority of the significant pairwise Fst/G'st scores (Table 4.7). There was no evidence of isolation by distance (F=1.64, p=0.212, df=2,8). Elevation of the surrounding landscape had no significant effect on Fst (r^2=0.03, p=0.555, F=1.67) or G'st (r^2=0.003, p=0.828, F=0.16).

Table 4.7. Pairwise Fst (below diagonal) and G'st (above diagonal), for populations in south Wales. Significant scores are denoted as follows: * p<0.05; ** p<0.01; *** p<0.001. Overall: Fst =0.0479, G'st=0.0791 both p<0.001, n=83.

	A (n=3)	B (n=18)	C (n=6)	D (n=7)	E (n=2)	F (n=17)	G (n=13)	H (n=8)	I (n=3)	J (n=2)	K (n=4)
A	-	0.0226*	0.0974*	0.1233**	0.1704	0.0711***	0.1164**	0.1162**	0.2824	0.2083	0.1378*
B	0.0933*	-	0.0248**	0.0091*	-0.0989	-0.0112*	-0.0130*	-0.0167	-0.005	0.0169	-0.0218
C	0.1284*	0.0739**	-	-0.0411	0.0554*	-0.0484	-0.0359	0.0048*	-0.0452	0.1724*	-0.0161
D	0.1783**	0.0584*	0.0416	-	-0.0592	-0.0382	-0.0815	-0.0351	0.0051	-0.095	-0.0225
E	0.1603	0.0327	0.1313*	0.0711	-	-0.1037	-0.1036	-0.1034	0.2561	0.3486	-0.0513
F	0.1568***	0.0250*	0.0236	0.0248	0.0565	-	-0.0393	-0.0257*	-0.0903	0.0201*	-0.0244*
G	0.1832**	0.0284*	0.0372	-0.0093	-0.0042	0.0075	-	-0.0474	-0.0481	0.0031*	-0.0851
H	0.1574**	0.0338	0.0662*	0.035	-0.0098	0.0340*	0.0126	-	-0.0602	-0.0352	-0.0652
I	0.2599	0.053	0.0444	0.064	0.1006	0.0138	0.0077	-0.0007	-	0.3521	0.0088
J	0.1891	0.0838	0.2350*	0.0007	0.1788	0.1414*	0.1155*	0.0642	0.2381	-	0.13
K	0.1696*	0.0514	0.0584	0.0564	0.0061	0.0635*	0.0105	0.0121	0.0293	0.1469	-

The easternmost colony, which forms area A in the analysis (Figure 4.7), shows significant divergence from the other colonies. Consideration of the topography and land use (EEA, 2018) shows that this colony is bordered by hills and urban development. This combination is likely to limit dispersal to and from the colony resulting in the genetic divergence of this population. This is most reasonably attributed to genetic drift given the low web count in some years (>35 in 2015 and 2016) (Butterfly Conservation, 2017c).

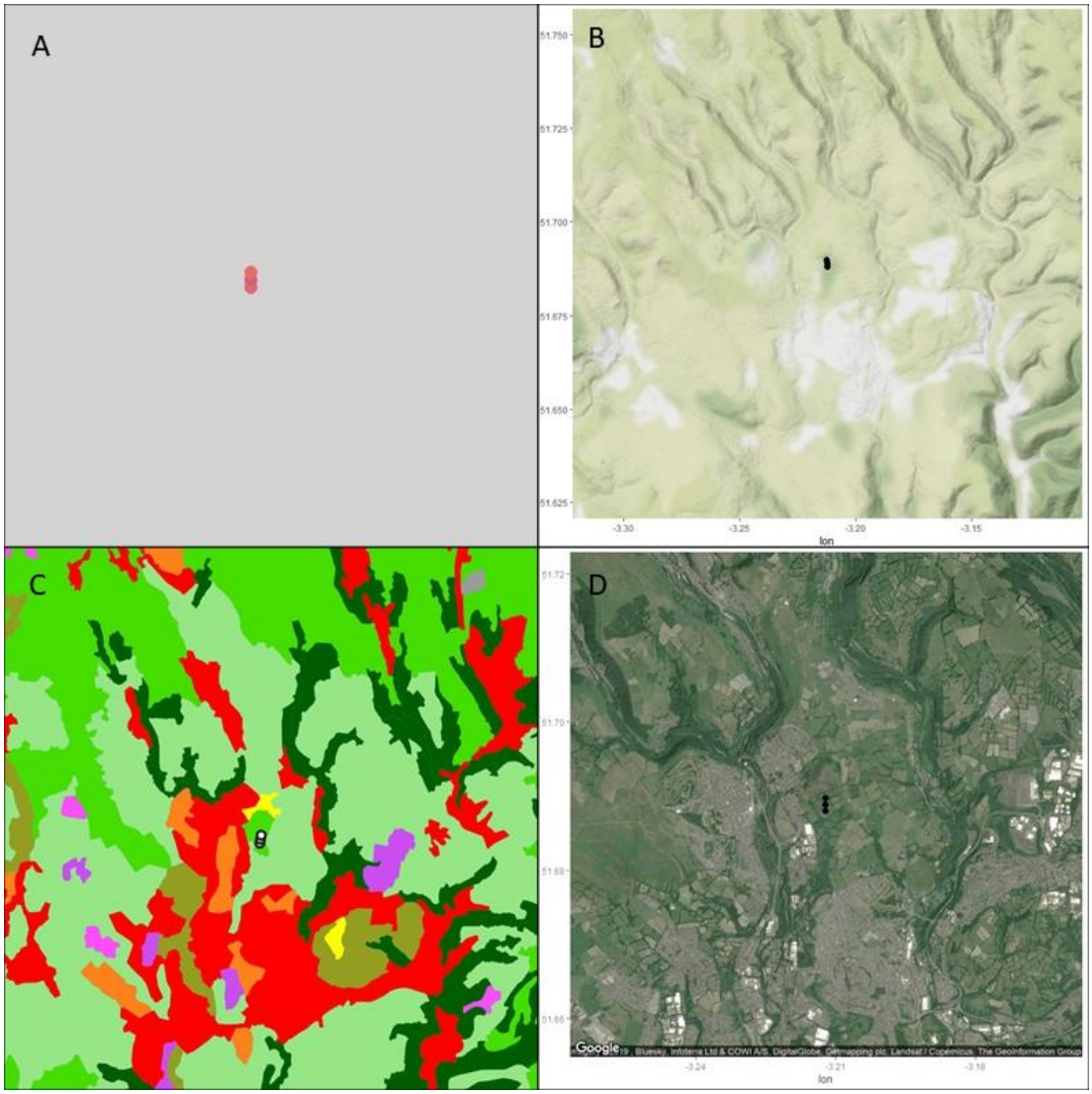

Figure 4.7. Mapping for isolated eastern colony, the colony is surround by urban development and hilly terrain. A) sPCA colorplot. B) Terrain. C) Land use (for colour codes see Figure 4.3). D) Arial imagery.

Area B consists of two colonies, these not only show divergence from each other and the rest of the region but also a remarkable amount of intra-colony variation (Figure 4.8). Examination of the topography and land use reveals flat pasture land between the two areas and aerial photography (Google Maps, 2019) does not show any obvious features which could prevent migration between the two areas.

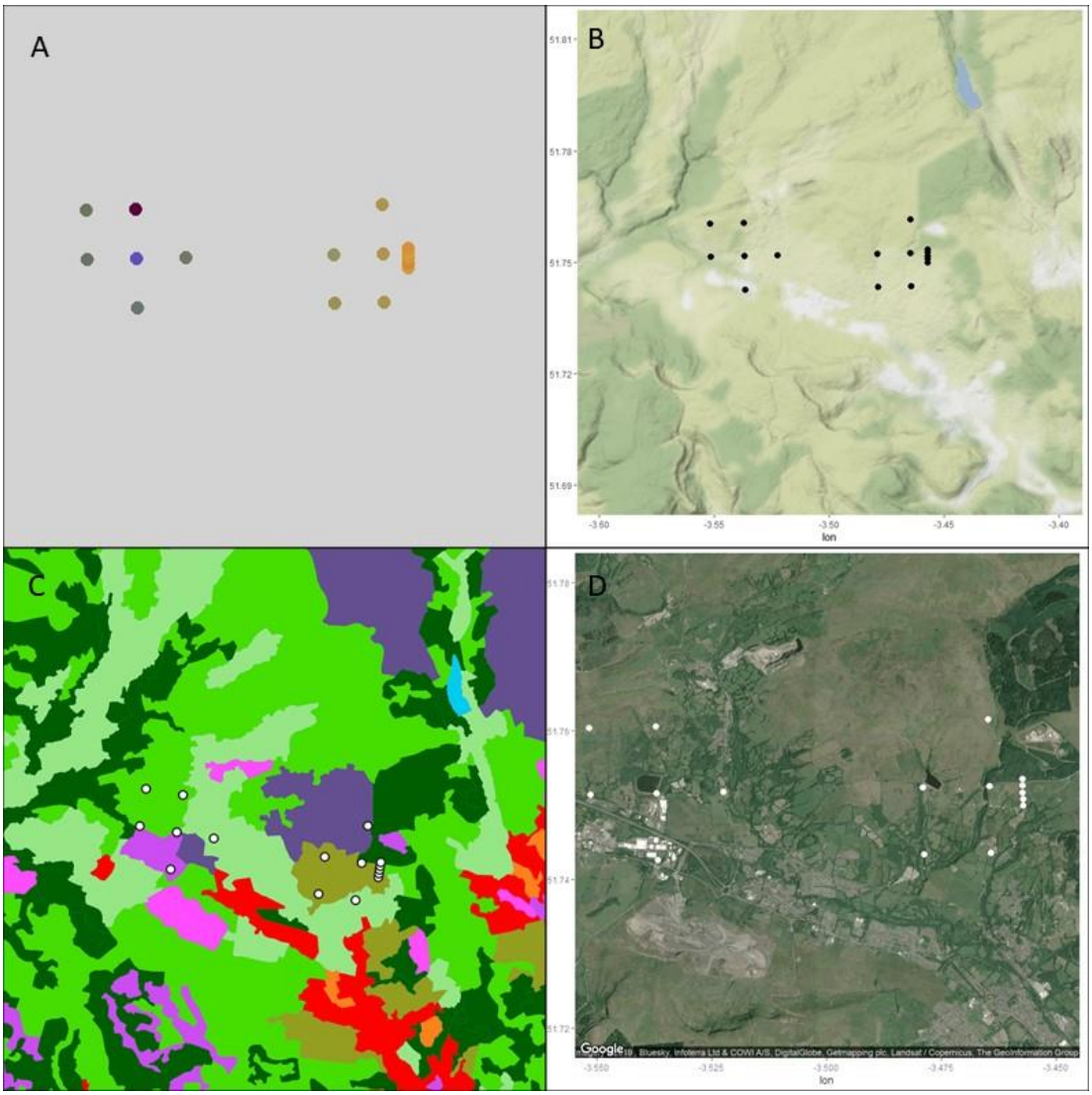

Figure 4.8. Mapping of genetically diverse close proximity colonies in area B, there is no obvious barrier between the areas. A) sPCA colorplot. B) Terrain. C) Land use (for colour codes see Figure 4.3). D) Arial imagery.

4.4 Discussion

The genetic information suggests that the four sites on the Lizard peninsula are not functioning as independent populations within the wider metapopulation. Instead there is one population in each area (Haylekimbro and Predannack) which has a patchy distribution with high levels of gene flow between the A and B patches within each area. This is unsurprising as the distance between the two patches within a site is less than 400m, well within the dispersal distance recorded by most studies (Warren, 1994; Sigaard *et al.*, 2008; Fric *et al.*, 2010; Zimmermann *et al.*, 2011; Konvicka *et al.*, 2012).

However it does contradict the assessment of Junker & Schmitt (2010) that the average lifetime movement is less than 100m and that life time movements over 300m are rare. Given that isolation by distance has been detected in this species (Chapter 2), this study suggests that the dividing point between patches and populations, at least in terms of geographical separation, lies somewhere between 0.44km and 3.39km, though other landscape features such as the nature of the matrix between patches will also affect this (Table 1.1). It is clear that the current management practice of treating the sites as four populations does not reflect the ecological situation of two populations.

Standard measures of genetic diversity for the Cornwall sites are similar to those of populations studied in Ireland and Scotland (Chapters 2 and 3, respectively). Although site Haylekimbro B does appear to have somewhat reduced diversity compared to the other three sites, this is likely to be a product of the low sample size (n=3). This is also likely to be the reason for the non-significant pairwise Fst and G'st between the populations at Haylekimbro B and Predannack.

Within south Wales the genetic diversity varies across the area studied. The eastern colonies display a greater level of differentiation, as shown compared with the

western colonies. The striking difference in differentiation between eastern and western areas may in part be explained by the topography of the region. The eastern part of the study is in a hilly region, with most peaks between 500-700m above sea level and with the colonies found in the valleys and lowlands. In contrast the western part is mostly low-lying and the colonies show less genetic variation, indicating probable higher levels of gene flow. The sole exception in the west is one colony in the north-west (area J) which is genetically distinct from its neighbours. However, this site is surrounded by low hills (~400m).

Despite the association between topographic isolation and genetic differentiation, the landscape analysis concluded that relatively high elevation between sites did not form a barrier to gene flow in the Marsh Fritillary. It is still possible that some other aspect of the topography is limiting gene flow between sites in hilly regions. In three species of Hesperid butterflies of the genus *Thymelicus* it has been shown that terrain is an important landscape component for gene flow between populations (Engler *et al.*, 2014). When aspects of topography and climate were evaluated, slope accounted for the largest proportion of the variation in population differentiation seen in *T. sylvestris*, and the second largest in *T. linola* and *T. action* (with the largest proportion of variation in these species accounted for by variations in land use). In all three species the slope and aspect of the site accounted for far more variation than did the altitude of the site.

Topography has been shown to have a strong impact on the microclimate of an area, slope and aspect in particular can impact the vegetation present on the surface as well as the near surface temperature and soil moisture content (Bennie *et al*., 2008). It is therefore reasonable to assume that a hilly region will have a more variable microclimate than a flatter region and variations in microclimate have been shown to impact the persistence of Lepidoptera (Weiss *et al.*, 1988; Suggitt *et al.*, 2015). An aspect of the topography of the hilly eastern region could therefore be

acting as a barrier to gene flow and yet not be detected when only altitude is examined. A study on the silver-spotted skipper (*Hesperia comma*) in the UK showed that variations in microclimate due to topography alter habitat associations at fine spatial scales adding further support to the idea that local variation in microhabitat within the hilly eastern region may be acting as a barrier to gene flow.

Climatic conditions have been shown to impact the movements of the *E. aurinia* complex in Italy by limiting the ability of some ecotypes to move between altitudes (Casacci *et al*., 2015). Similarly Botham *et al*. (2011) found that elevation impacted upon the presence of the larval host plant via competitive exclusion at both the upper and lower ends of the 200m altitudinal range investigated. Studies on other Lepidoptera species have demonstrated that microclimate, which is affected by slope, incline and aspect, can have a significant effect on larval development (Weiss *et al*., 1988).

The lack of isolation by distance (IBD) is consistent with the findings in Scotland (Chapter 3) but contradictory to the findings in Ireland (Chapter 2). This suggests that there may be a consistent pattern in the Marsh Fritillary where IBD is a factor only above a certain separation distance. Isolation by environment (IBE) is the process whereby environmental features form barriers to dispersal resulting in population structuring (Wang & Bradburd, 2014). This has been detected in the butterfly *Archaeoprepona demophon* where canopy position was found to be an isolating factor. It is also possible for IBD and IBE to interact, a review by Sexton *et al*. (2014) across a broad taxonomic range found evidence of the combined effect of IBD and IBE in 37.1% of the studies reviewed. When taxonomic groups were considered separately, 25.0% of invertebrate studies showed both IBD and IBE (16.7% IBD only, 41.7% IBE only). Therefore, in the Marsh Fritillary it may be that for separation distances of <100km IBE is the more important factor while at distances

>100km IBD becomes the more influential factor, based on the detection of IBD in Ireland (Chapter 2) but not in this chapter.

With regards to Area B and the genetic variation within the area, shown by the distinct colours within the area on the sPCA, it is possible that this area has been recently colonised (or recolonised following a localised extinction). This possibility is supported by records which indicate that during the period of collection (2011-2012) Marsh Fritillary numbers were increasing across Wales generally (Butterfly Conservation, 2019). Under this scenario the areas were colonised by the initial founders and then several more migrants arrived at a later date, accounting for the divergent samples in each area. These arrivals would have been within the past couple of generations to account for the lack of intermediate genotypes between the founders and recent immigrants. Of interest to note is that the samples for the most western of the two areas were collected in both 2011 and 2012 (eastern site 2011 only). The divergent samples identified by differing colour in the sPCA, came from the second year. It is tempting to speculate that their parent may have arrived that year or a grandparent the year before. It is also possible, given the divergent genotypes, that some of the founders and later migrants came from colonies and sites not included in this study.

Based on the genetic information of this study the following management recommendations can be made; the four sites on the Lizard peninsula should be managed as two separate populations and, in each area, efforts be made to increase the patches of Devil's-Bit Scabious available to provide additional habitat and increase the carrying capacity of the population. This will improve the likelihood of persistence for each population. In South Wales, areas A and B contain genetic variation not represented elsewhere in the region and should be the focus of additional conservation attention to maintain the populations in these areas. This is also

applicable to part of area J which warrants further investigation as it is possible that the unusual sample is representative of additional unique genetic variation outside of the geographical scope of this study.

The finding of this combined study has implications for those working on the conservation of the Marsh Fritillary. Firstly, that sites in close geographical proximity (<500m) are likely to be patches of the same population. Secondly that genetic diversity is not evenly distributed across the landscape and consideration of this needs to be made when planning conservation management, including the selection of priority sites and possible sources for reintroduction attempts. It would also be worth undertaking a more sophisticated landscape analysis that incorporates the distribution of the larval food plant as well as other landscape features such as aspect, temperature and incline, to better understand the landscape features which impact gene flow.

5 There and back again: Assessing the genetic diversity and composition of the reintroduced Marsh Fritillary population in Cumbria.

5.1 Introduction

Reintroduction is defined by the IUCN Species Survival Commission Reintroduction Specialist Group (2013) as “the intentional movement and release of an organism inside its indigenous range from which it has disappeared”. Their guidelines (Table 5.1) stress the importance of understanding the biological and ecological requirements and interactions of a species. For this reason, there are a limited number of species which are suitable for reintroduction due to a lack of information on the basic requirements of a species. It is critical to understand the processes that caused the extinction, as well as the ecology and life history of the species to be reintroduced (Sarrazin & Barbault, 1996; Armstrong & Seddon, 2008).

Table 5.1 Criteria for assessing the suitability of a species reintroduction plan (IUCN/SSC, 2013). In cases where detailed biological and ecological knowledge is not available for an endangered species information from a closely related species may be substituted.

Criteria	Subcategory
Biological feasibility	a. Basic biological knowledge of the species b. Habitat requirements c. Climatic requirements d. Founders e. Animal welfare f. Disease and parasite considerations
Social feasibility	a. Local communities b. Other translocation projects c. Ecological and human social balance
Regulatory compliance	a. International b. National c. Regional d. Sub-regional
Resource availability	a. Funding availability b. Biological/technical expertise

Despite the challenges it presents, reintroduction and related translocation practices, such as reinforcement (adding individuals to an existing population) and assisted colonization (establishment of individuals in suitable habitat outside of the species native range as protection from anthropogenic threats, usually climate change) are becoming increasingly common in conservation practice (Seddon *et al*., 2007; 2012). The IUCN have published 349 reintroduction case studies covering all major taxonomic groups (plants (59), invertebrates (28), fish (35), amphibians (20), reptiles (37), birds (64) and mammals (106)) (Soorae, 2018). However, there is a definite taxonomic bias in favour of vertebrate animal species, notably mammals and birds, possibly for the simple reason that they are better understood and attract better funding. This has led to concern that the focus on larger charismatic species may be diverting limited conservation resources away from taxa which are in greater need of attention (Seddon et al., 2005; Colléony *et al.,* 2017).

Although the success of reintroduction can be measured in a number of ways, the primary measure is generally the establishment and successful breeding of a population. Typically this is only monitored for a few years or to the birth of the F1 or F2 generation (Wauters *et al*., 1997; Spalton *et al*., 1999; Richards & Short, 2003; Sarrazin *et al*., 2008; Godefroid *et al*., 2011; Sanz & Grajal, 2010). One aspect of success less commonly considered are genetic measures of population stability, and when they are assessed there is a heavy bias to studies of birds and mammals (Groombridge *et al*., 2012).

As indicated in the IUCN Guidelines, genetic composition of the founder stock is of critical importance to a reintroduction effort (IUCN/SSC, 2013). As a species declines towards extinction it will experience a genetic bottleneck, the extent of which will be determined by the individual species' decline pattern (Groombridge *et al.*, 2012). Populations which are reintroduced may experience an additional bottleneck at the time of reintroduction and potentially a third where the reintroduction is from

captive bred stock, as the founding of the captive breeding population is itself a bottleneck (Groombridge *et al*., 2012).

For UK Lepidoptera, the most publicised successful reintroduction was that of the Large Blue butterfly (*Phengaris* (=*Maculinea*) *arion*). It became extinct in the UK in 1979 due to a combination of changing farming practices, the sharp reduction in rabbit numbers due to myxomatosis and a poor understanding of the specificity of *P. arion*'s dependence on *Myrmica* spp. (red ant) hosts. It was known that *P. arion* was a brood parasite of *Myrmica* spp. however it wasn't recognised that it required a specific species, *Myrmica sabuleti* (Muggleton & Benham, 1975; Thomas *et al*., 1989). Changes in agriculture, including abandonment, caused an increase in sward height which allowed other *Myrmica* species to outcompete *Myrmica sabuleti* and caused the decline of *P. arion.* Prior to the first attempt at reintroduction in 1984, sites were managed, including appropriate grazing and/or mowing regimes, to encourage *Myrmica sabuleti.* As the UK endemic subspecies (*P. a. eutyphron*) was extinct, the pilot reintroduction used stock from Sweden (*P. a. arion*), judged to be the most ecologically similar. The pilot was successful and was followed by further reintroductions in 1986. The species is currently considered to be established and breeding in the UK (Thomas *et al*, 2009).

To date there have been seven recorded extinctions of British butterflies (Table 5.2) and 70% of species have decreased in occurrence between 1976-2014. Over the last ten years 52% of UK butterfly species have decreased in abundance (Fox *et al*., 2015). For species which are declining but not yet extinct, reintroductions are a critical method of restoring their historical range and offering increased resilience against stochastic events. Translocations can also increase the genetic diversity of small or isolated populations. This can be as part of a captive breeding program, the release of individuals through population reinforcement or the creation

of intermediary stepping-stone reintroduced populations to establish natural gene flow between isolated populations.

Table 5.2. Butterflies recorded as extinct in Britain and Ireland. References: [1]Ford (1945), [2]Emmet & Heath, (1990), [3]Thomas & Lewington (1991), [4]Asher *et al*. (2001), [5]Fox *et al*., (2006).

Name	Extinction Date	Extinction cause (known or suggested)	Notes	Ref
Lycaena dispar **(Large Copper)**	1851	Habitat loss Over collection	Attempted reintroductions with only short-term success presently extinct	1, 3, 4
Cyaniris semiargus **(Mazarine Blue)**	c. 1904	?	Occasional single specimens seen up to 1958, likely migrants from Europe, no known breeding in British Isles	1, 3, 4, 5
Aporia crataegi **(Black-veined White)**	c. 1925	?	Failed reintroductions before and after extinction. Occasional vagrant adults seen	1, 3, 4, 5
Phengaris (=Maculinea) arion **(Large Blue)**	1979	Management changes severely affected host ant *Myrmica sabuleti*	Successfully reintroduced 1984. 2004, established at multiple sites	4, 5
Carterocephalus palaemon **(Chequered Skipper)**	1976 (England only, stable in Scotland)	Habitat loss	Attempted reintroduction to England in 1990s failed due to lack of habitat. Scottish population not recorded by Ford.	1, 2, 3, 4, 5
Erebia ligea **(Arran Brown)**	?	?	First recorded 1800-1810, authenticity of specimens is debated. Recorded again in 1969, not believed to be migrant or vagrant, current status is unknown	1, 2, 5

Documentation of reintroductions is becoming increasingly common in the scientific literature, including monitoring (Wakamiya & Roy, 2009; Bernardo *et al*., 2011; Nichols & Armstrong, 2012), management (Jones & Merton, 2012; West *et al*.,

2017), range expansion (Halley *et al*., 2012; Gaywood, 2018) and population modelling (for summary see Armstrong & Reynolds, 2012).

Considerably less well explored are the changes that may occur to the genetic diversity of the reintroduced or supplemented population over the generations following the intervention. Such studies have tended to focus on comparisons between reintroduced and captive populations (Alcaide *et al*., 2010) and inference of historic genetic patterns from modern populations (Biebach & Keller, 2009). However, such studies are important for the ongoing success of a reintroduction as they can indicate if further management is needed. Such a case was observed in the black-footed ferret (*Mustela nigripes*) where reintroduced populations which did not receive additional augmentation after the initial release displayed reduced allelic diversity and changes in phenotype including an overall smaller body size and shorter limbs. There was also a surprising level of population differentiation detected given the common source population. These changes occurred within 10 years of the reintroduction and it was recommended that limited translocations be instituted to counter this reduction in genetic diversity (Wisely *et al.*, 2008).

A similar reduction in genetic diversity following reintroduction was observed in the American marten (*Martes americana*) reintroduced to Michigan. 20-25 years after reintroduction the populations displayed moderate levels of inbreeding and low allelic richness, which is attributed to the small number of initial founders (85) resulting in an even lower effective population size (calculated to at times have been as low as 6 individuals) There was also a significant level of population structuring detected between the release sites (Hillman *et al.*, 2017).

Other investigations of the genetics of reintroduced populations have produced more hopeful results. A study of the genetics of reintroduced eastern wild turkeys (*Meleagris gallopavo silvestris*) revealed evidence of gene flow between reintroduced populations, this was based on the assignment of individuals, using

mitochondrial haplotypes and microsatellite genotyping, to source populations not used to found the population in which they were living. This suggests that the reintroduced populations were incorporating into the wider landscape population structure of the species in the area (Latch & Rhodes, 2005). Another study assessed reintroduced populations of the Griffon vulture (*Gyps fulvus*) across Europe and concluded that genetic diversity of natural and reintroduced populations was similar (Le Gouar *et al.*, 2008). This was attributed to maintenance of genetic diversity in the captive stock which supplied the founders for the reintroduction. However, there is a significant taxonomic bias in the literature towards bird and mammal species, and relatively few papers address the population genetics of reintroduced invertebrate species.

Dolný et al. (2018) compared the population genetics of source and destination populations of White-faced darter (*Leucorrhinia dubia*) 14 years after the reintroduction, they found a moderate level of differentiation between the two sites at the time of the study. The reintroduced population had a higher allelic richness than did the source population, possibly due to habitat loss and population degradation at the source since the translocation was carried out.

Andersen et al. (2014) compared the reintroduced populations of the Large Blue butterfly (*P. arion*) with the source population in Öland, Sweden, and other populations in Scandinavia. 19 years after reintroduction the UK populations were found to have similar levels of genetic diversity to both the source population and to other Scandinavian populations, however the study also found a significant change in allele frequencies and that there was significant population differentiation between the UK reintroduction sites.

The Marsh Fritillary butterfly (*Euphydryas aurinia*) is a univoltine species found throughout much of Europe. Although presently classified as Least Concern, reflecting its broad geographical range, it is declining throughout its range and

regionally extinct in certain areas. The British Isles has been identified as a stronghold for the species, possessing up to 15% of the European population. It is legally protected under the EU Habitats Directive and, since 1998, the Wildlife and Countryside Act 1981.

Despite legal protections and conservation efforts it has still undergone significant declines in Britain over the past thirty years. This is believed to be due to changes in agricultural practices, including enrichment of pastures (Porter & Ellis, 2011), leading to a decrease in suitable habitat for Devil's-bit Scabious (*Succisa pratensis*) (Ridding *et al*., 2015), the larval food plant, which has been shown to be a key predictor of Marsh Fritillary occurrence (Brunbjerg *et al*., 2017)

The populations of the Marsh Fritillary in Cumbria present in interesting case study to allow the examination of the changes in genetic diversity following reintroduction. The history of the Marsh Fritillary in Cumbria has been well documented (Ford & Ford, 1930; Ford, 1945; Porter & Ellis, 2011). Museum and survey records indicate the Marsh Fritillary was previously widely dispersed throughout Cumbria; it was found as far east as Castle Carrock in the North Pennines and throughout Cumbria as far south as Ennerdale (Figure 5.1). The occurrence of records in Dumfries suggests that what is called the Cumbrian population once extended far beyond the boundary of the county. The population has been described as experiencing cyclic population increases and decreases (Ford & Ford, 1930).

However from the 1980s it began to decline irreversibly and increasing local extinctions were noticed (Porter & Ellis, 2011). Despite intensive efforts, including improved management of known sites and increased searching for new colonies to implement management practices, the decline could not be halted. In the late 1990s there were two separate attempts to release additional individuals to the area but both introduced colonies failed in their first year for unknown reasons, though a low release

number and lack of suitable pre-release site management are suspected to have played a role (Porter & Ellis, 2011).

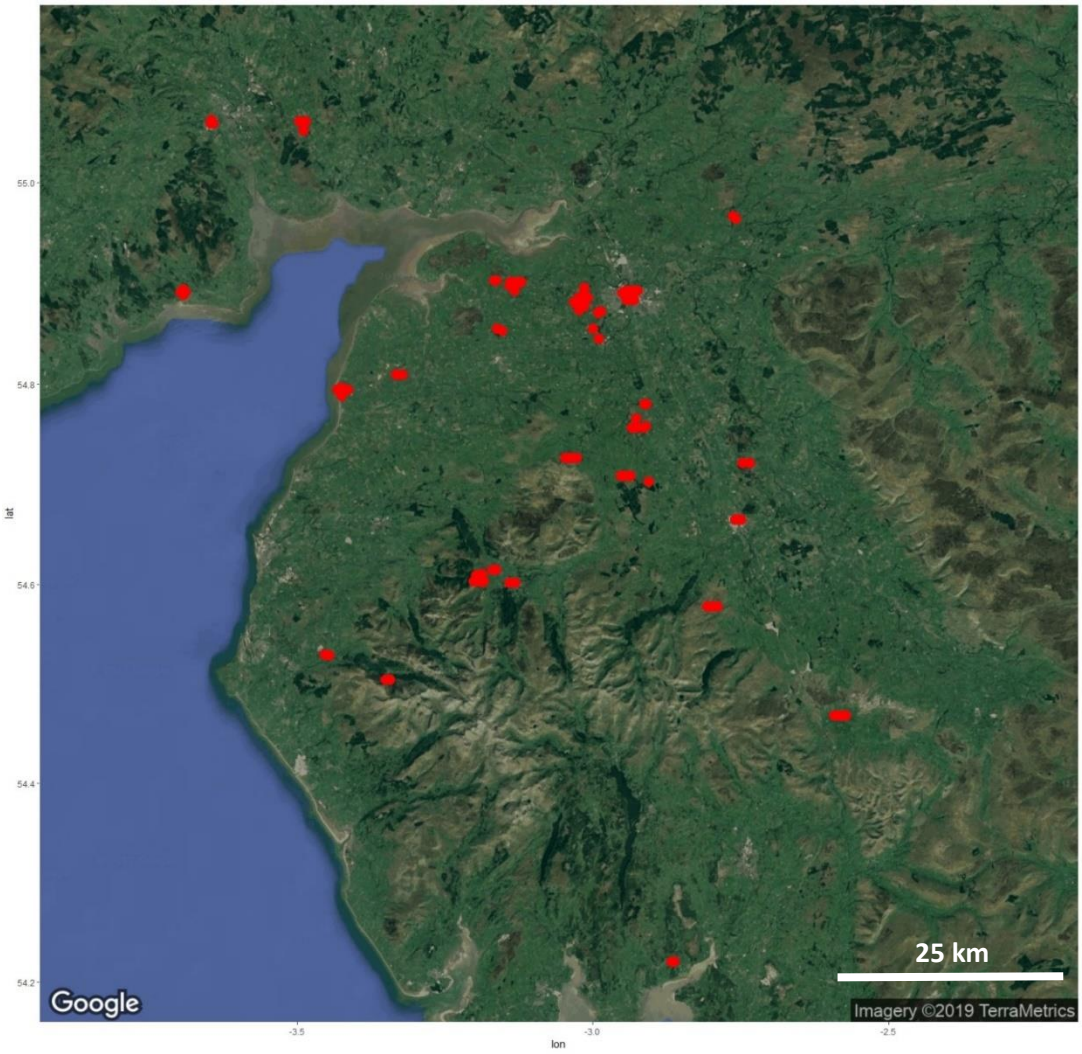

Figure 5.1. The historic distribution of the Marsh Fritillary in the Cumbria area based on pre-2005 records. Red circles represent approximate location of a record, one location mark may represent several records. Combined datasets from NBN Atlas (2019) & GBIF (2019)

By 2004, one population remained in Cumbria and only a single female was seen. Extensive surveying in autumn 2004 found just one larval web and the decision was made to take the larvae into captivity with the intention of future reintroduction. As the larvae were the offspring of a single female and the male which mated her, there was a concern that the resulting captive population would suffer from inbreeding depression, particularly given that the wild population had been contracting for several

years and therefore there was a strong chance that a level of inbreeding had already occurred. The decision was made to outcross the captive population to individuals collected from the Argyll region of Scotland due to the suggestion of historic gene flow between the populations (Porter & Ellis, 2011), 95 larvae from 19 sites across the Argyll region (five larvae per site) were collected for this purpose.

This admixture population flourished in captivity and in 2007 the first reintroductions were carried out. The four chosen sites were prepared to suit the requirements of the Marsh Fritillary, including the institution of suitable grazing or mowing regimes and the planting of additional *S. pratensis* (for details see Porter & Ellis, 2011). 42,000 5/6th stage instar larvae were released across the four sites (~10,000 per site), with ~8,000 retained in captivity as insurance against failure and for future reintroductions at additional sites if the initial attempt was successful.

The reintroduction was successful as all four reintroduction sites established and persisted (Porter & Ellis, 2011). Since 2007 additional reintroductions and natural colonisations mean that several currently successful metapopulations are established in Cumbria (Porter, pers. comms). However, the genetic composition of these metapopulations is unknown. Although none of the founders of the captive population were preserved, there are historic specimens held in museums and private collections which date from both the pre-decline period (1920-1930s) and the final decline of the original Cumbrian populations (1970-1993). These historical specimens along with specimens recently collected in Scotland from the areas where the Scottish founder stock originated will allow for a comparison between the reintroduced admixture population and the founder source populations.

The aim of this study is to assess the genetic composition of the reintroduced populations to determine: 1) If the populations at the four original reintroduction sites have similar levels of genetic variation compared with natural populations, 2) to compare the genetic diversity of the present Cumbrian population with the historic one

and with the Scottish donor populations, and 3) to determine if there is any genetic differentiation between the four reintroduction sites after nine generations.

This is the first time that a study of this type has been carried out in Lepidoptera, a comparable study on the Large Blue compared the reintroduced UK population with the Swedish donor population (Andersen *et al*., 2014). In this study, however, the comparison is of an admixture population with the two donor populations, one of which was the original population at the reintroduction site. This before and after comparison is, to the best of the authors' knowledge, unique in the Order.

5.2 Method

5.2.1 Site selection and field sample collection.

The sites used in this study are the four reintroduction sites described in Porter & Ellis (2011) (Figure 5.2 & Table 5.3), these sites were selected as they were the oldest of the reintroduction sites and had been established in the same year. In addition, some later reintroductions at other sites were carried out after the captive stock had been 'refreshed' with larvae collected from established reintroduction sites (Porter, pers. comm.) which would complicate analysis of population differentiation.

Larvae were collected August 2016 larvae were collected (under Natural England licence number 2016-25165-SCI-SCI. A single larva per web was collected to prevent the collection of closely related individuals. A maximum of 30 larvae per site were collected as this was judged to give a fair representation of the genetic diversity at each site (Hale et al., 2012; Smee, 2011).

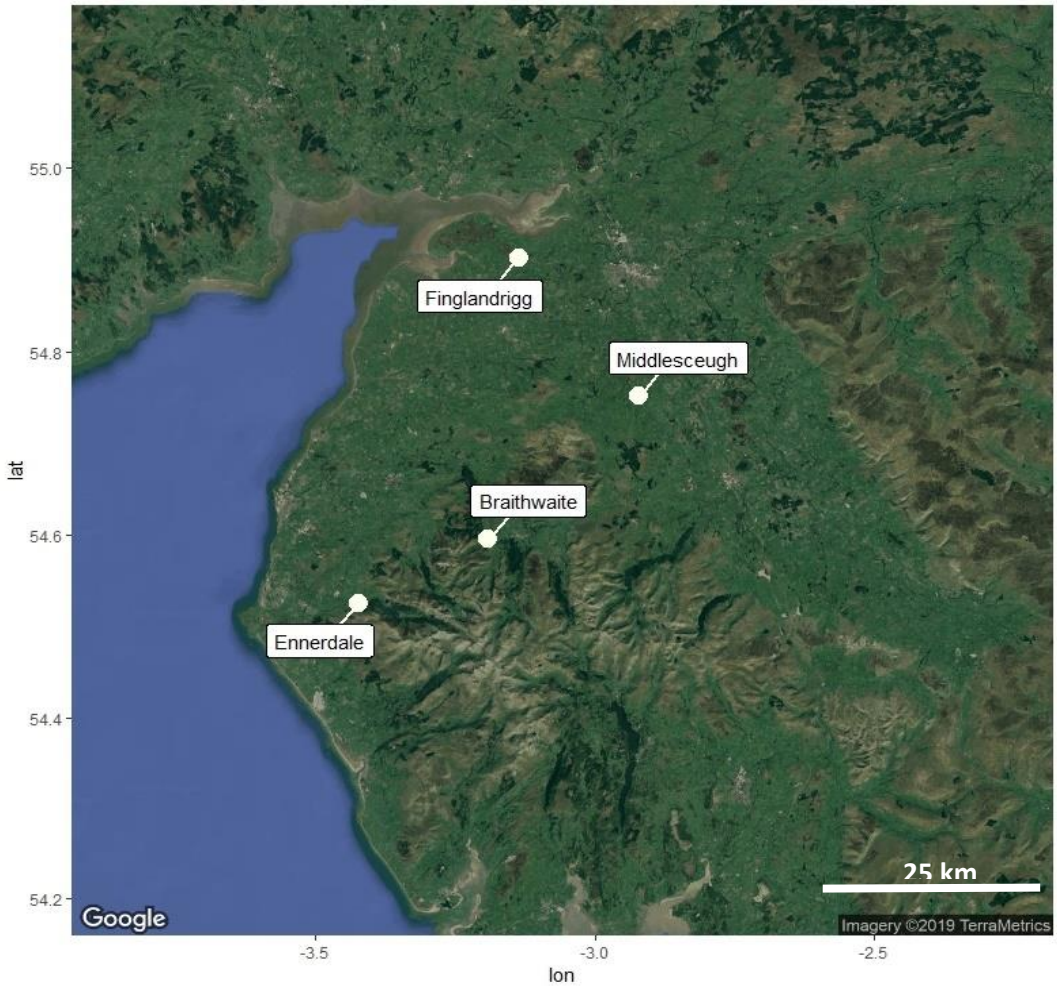

Figure 5.2. The sites of the reintroduction as described in Porter & Ellis (2011) which were sampled as part of this study.

Larvae were collected from Scotland in August and September 2017 (under Scottish Natural Heritage licence number 104772) from four sites in the Argyll region (Figure 5.3) (for full site details See Chapter 3). It was not possible to sample the exact colonies used as donors due to the time that had elapsed since they were collected, however the broad geographical areas were known and two populations within these areas (Tayvallich and Mull) were samples to represent the original Scottish donors. Two additional populations within the Argyll region which are not suspected to be donors (Islay and Oronsay) were also sampled for comparison. Collection was carried out as in Cumbria.

Table 5.3. Site information for sampling sites In Cumbria as described in Porter & Ellis (2011). Note that at the request of the Cumbria Marsh Fritillary Action Group exact locations and site descriptions are not provided. Regional climate information: Mean annual maximum temperature 13.0°C, mean annual minimum temperature 5.8°C, annual rainfall 1521.0mm (for detailed climatic data see Appendix C). Surrounding land use taken from EU CORINE land use data set, underlined land use is land use of site, other listed are land uses of surrounding area. Geological information form British Geological Society.

Site	Location	Elevation (m)	Surrounding land use	Bedrock geology	Sediment geology
Braithwaite	54.59 / -3.19	188	Moors and heathland, Pastures	Kirk Stile Formation - Mudstone and Siltstone.	Talus - Rock Fragments, Angular, Undifferentiated Source Rock.
Ennerdale	54.52 / -3.42	298	Moors and heathland, Natural grasslands, Pastures, Transitional woodland-shrub, Mixed forest	Ordovician Rocks (undifferentiated) - Mudstone, Siltstone and Sandstone.	Alluvial Fan Deposits - Sand and Gravel
Finglandrigg	54.90 / -3.13	14	Broad-leaved forest, Pastures, Non-irrigated arable land	Mercia Mudstone Group - Mudstone with Gypsum-stone and/or Anhydrite-stone	Peat - Peat.
Middlesceugh	54.75 / -2.92	167	Pastures, Broad-leaved forest, Non-irrigated arable land	Stainmore Formation - Mudstone, Sandstone and Limestone.	Till, Devensian - Diamicton.

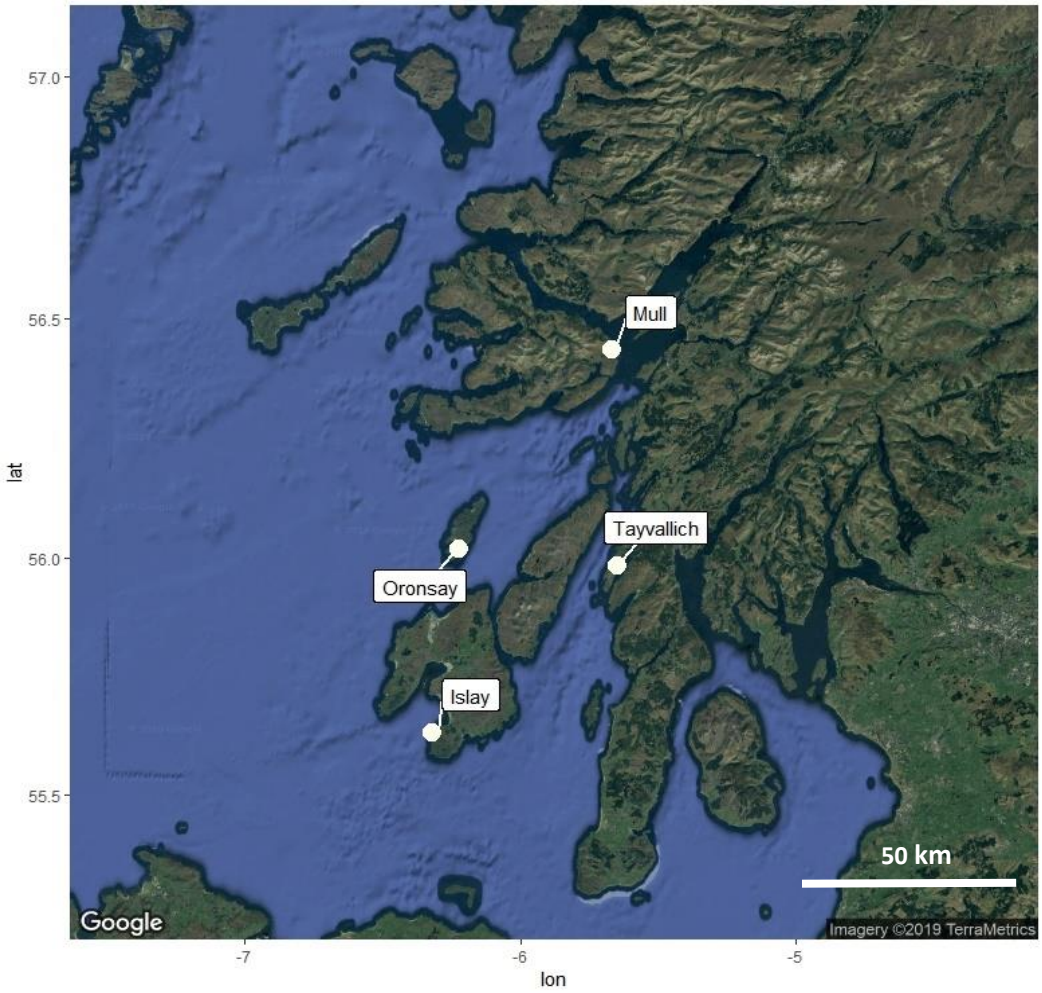

Figure 5.3. Sampling sites in Scotland, Tayvallich and Mull were among the 19 donor sites for the captive breeding projects, Islay and Oronsey were not.

Historic specimens from the 1920-1930s held in museum collections (here after designated Cumbria Historic), and specimens from the 1970-1990s held in a private collection (here after designated Cumbria Donor), were sampled by removal of a leg with entomological forceps with permission from the collectors or museum curators. These specimens were assigned to a geographical population based on the location information provided with the specimen (Figure 5.4).

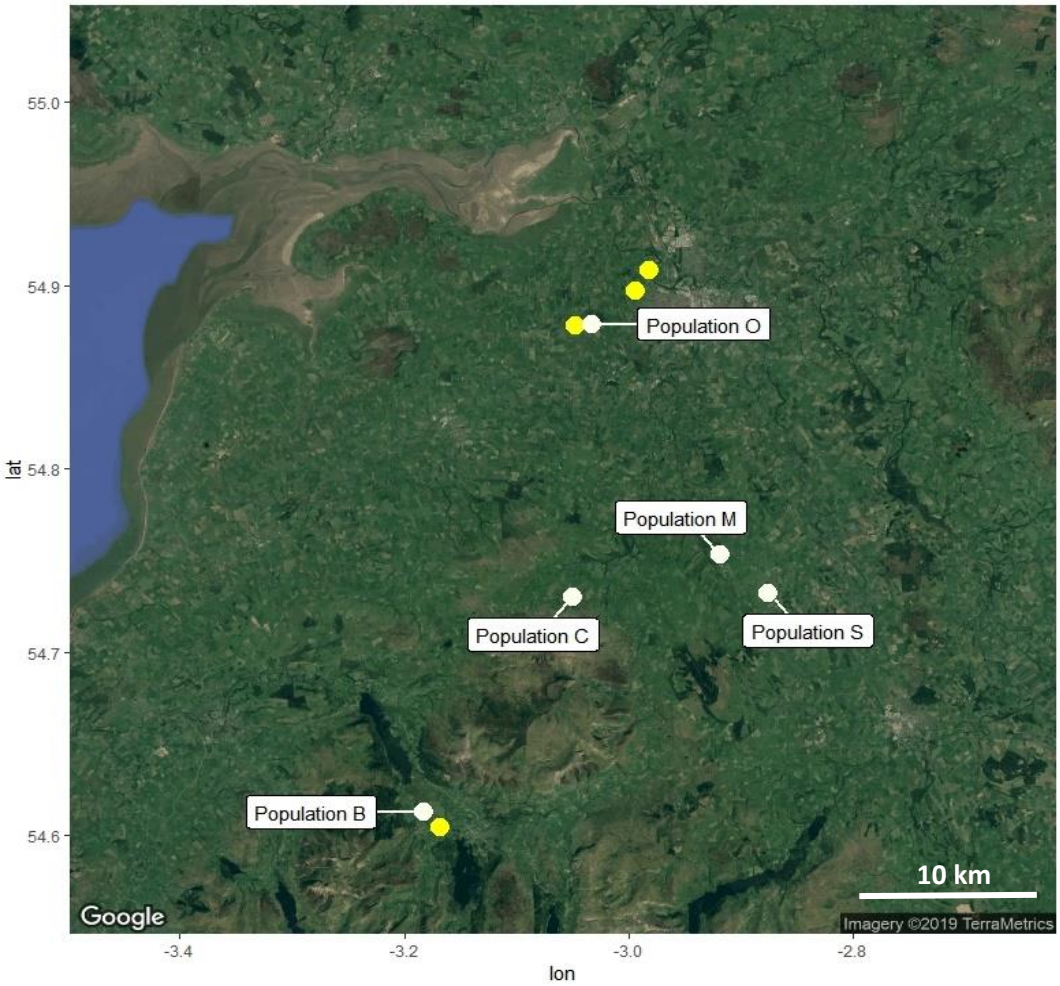

Figure 4. Sites at which preserved samples were collected, multiple samples were collected at most points. Yellow points are 8 specimens collected 1920-1930. 20 specimens collected 1970-1990s were assigned to five geographical populations based on proximity.

4.2.2 Laboratory work and analysis

Specimens were stored at -80°C until used. Larval heads were used as the source and DNA was extracted using DNeasy Blood and Tissue kit (Qiagen), following the standard protocol supplied, and extracted DNA was frozen at -20°C until use. For museum samples the removed leg was used as the source DNA extraction was carried out as for modern samples but with increased lysis time (12hr instead of 3hr).

Microsatellites which had previously been developed for the Marsh Fritillary were used: Aurinia_01, Aurinia_13, Aurinia_45, Aurinia_70, (Smee *et al.*, 2013) and

Eau88 (Sinama *et al*., 2011). These were amplified using the protocol in Appendix A. PCR products were separated via capillary electrophoreses on an AB3500 Genetic Analyser (Applied Biosystems) and sized relative to an internal size standard (LIZ500; Applied Biosystems) using GeneMapper 5. Sizes were checked manually and individuals with unclear peaks were amplified and genotyped again. Due to degraded nature of the samples, slightly greater leeway was allowed when genotyping preserved collection specimens, provided that clear peaks could be identified (Appendix A). Raw allele scores were binned using TANDEM to reduce error in the binning process.

Analysis was carried out in R (version 3.3.2) using binned allele sizes unless otherwise stated, no transformations of the data was carried out unless otherwise stated. To characterise the genetic variation within populations basic population statistics were calculated using diveRsity (Keenan et al., 2013). Pairwise Fst and Nei's G'st were calculated with corresponding p-values were calculated using strataG (Archer *et al.*, 2017) to determine the level of differentiation between populations. Principal Component Analysis was used to analysis genetic structure of the reintroduced population with reference to the donor populations, this has been demonstrated to be a suitable analysis technique in admixture populations (Ma & Amos, 2012). The analysis was carried out in `Gstudio` (Dyer, 2012) and visualised with `ggplot2` (Wickham, 2009).

The statistical power of the microsatellites used was assessed in POWSIM 4.1 (Ryman & Palm, 2006), this used allele frequencies for the total dataset to carry out a Fisher's exact test with 10000 replicates.

5.3 Results

Power analysis showed that, based on detected allele frequencies and under a conservative estimate of an effective population size of 1000 individuals (assuming all samples are unrelated), the microsatellites used would be able to detect Fst of 0.01 in 81% of cases and Fst of 0.02 in all cases which was deemed sufficient to answer the research question.

5.3.1 Reintroduced population genetic diversity.

All sites showed significant heterozygote deficiency (Table 5.3). Private alleles were observed at each site, accounting for 7.14-10.71% of observed alleles at a regional level. No significant level of inbreeding (Fis) was detected. Pairwise Fst values (Table 5.4) are mostly significant, the exception is between Braithwaite and Middlesceugh which is non-significant. G'st values, which are standardised to take into account variation in diversity at different loci, show a similar pattern to the Fst scores.

The Principal Component Analysis (Figure 5.5) showed no clear separation between all four reintroduced populations. However, Middlesceugh shows little overlap with Finglandrigg, Middlesceugh also shows little overlap with Ennerdale. Braithwaite shows extensive overlap with both Finglandrigg and Ennerdale.

5.3.2 Comparison with founder populations.

The museums specimens from 1920-1930 cluster largely within the 1970-1990s (Figure 5.6). There is almost complete separation between the reintroduced and all others on the PC1 axis. Only three disparate reintroduced samples lie to the west of the most easterly historical specimens. In addition, a sample from both Ennerdale and Middlesceugh lie close to the historic specimens.

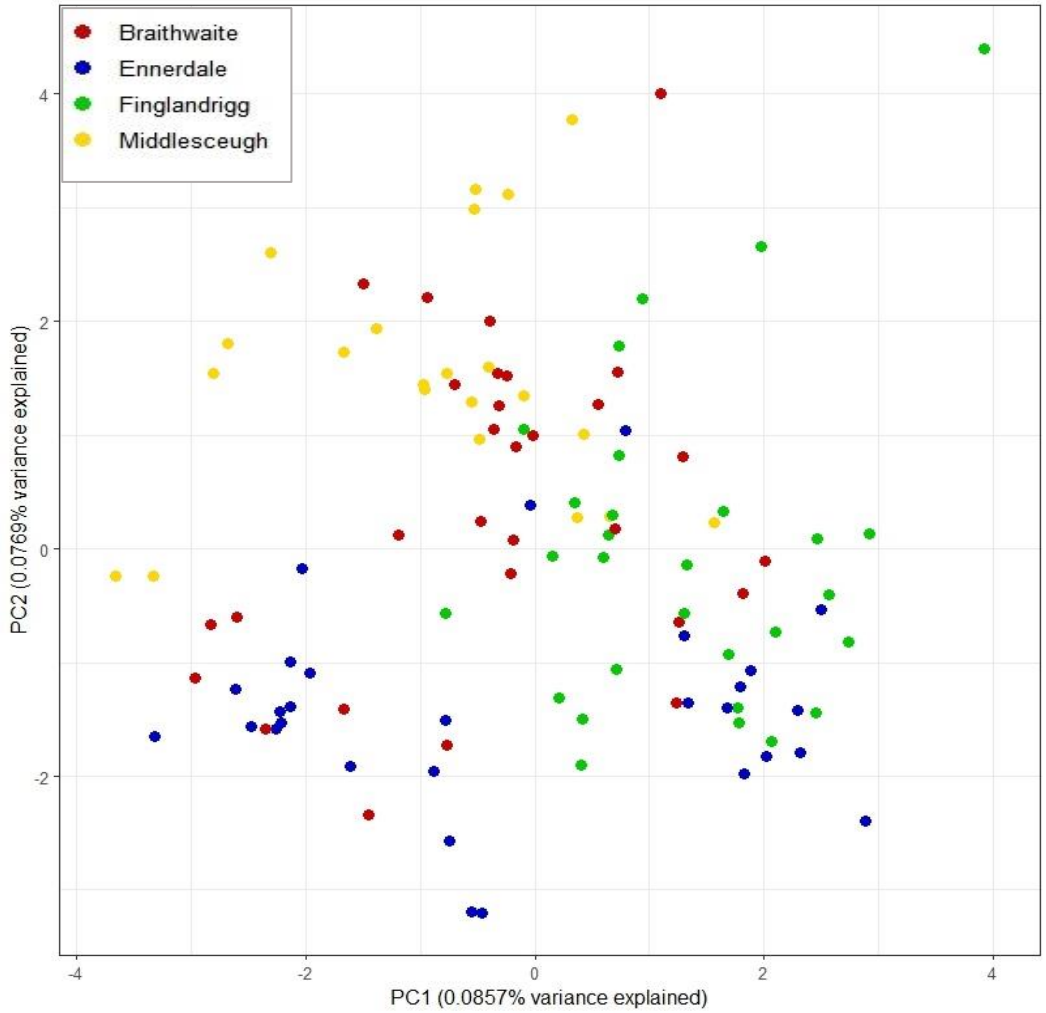

Figure 5.5 Principal component analysis for the reintroduced sites in Cumbia, each point represents an individual collected from that site.

The Cumbrian reintroduction sites show higher allele number, richness and Fis compared with Scotland (Table 5.3). Scotland shows a higher occurrence of private alleles than Cumbria, however Cumbria does contain private alleles which are not present in the Scottish populations. The historical Cumbrian population also contains private alleles not present in the modern population or Scotland. When the two historic time frames are considered separately, the later population (1970-1990s) does not contain any private alleles while the earlier time period (1920-1930) does.

Table 5 3. Genetic diversity statistics for Cumbrian reintroduction sites, Cumbrian historical populations (including both time periods) and Scottish sites (non-donor sites in italics), mean values across all loci shown. Allelic richness is calculated using 1000 resamples (n=smallest input sample size), with replacement per populations. Private alleles are given as the percentage of that regions alleles across all loci found only in that population. H_e expected and H_o observed heterozygosity. *When considered separately the 1920-1930 population has10.52% private alleles not found in another study population while the 1970-1990 population did not contain any private alleles

Region	Site	Sample size	Allelic richness	Private alleles (%)		Ho	He	Fis (95%CI)
				Region	Total			
Cumbria	Braithwaite	30	5.41	9.67	6.45	0.43	0.63	0.310 (0.186, 0.434)
Cumbria	Ennerdale	29	4.87	10.71	10.71	0.38	0.55	0.297 (0.156, 0.429)
Cumbria	Finglandrigg	30	4.65	7.4	7.40	0.49	0.56	0.114 (-0.020, 0.251)
Cumbria	Middlesceugh	23	4.84	7.14	3.57	0.51	0.61	0.172 (0.003, 0.316)
Scotland	*Islay*	*22*	*2.89*	*0*	*0*	*0.44*	*0.43*	*-0.021 (-0.180, 0.137)*
Scotland	Mull	12	3.18	11.11	5.55	0.47	0.52	0.096 (-0.169, 0.326)
Scotland	*Oronsay*	*30*	*3.58*	*16*	*8.00*	*0.42*	*0.51*	*0.166 (0.036, 0.289)*
Scotland	Tayvallich	30	4.19	19.23	15.38	0.52	0.63	0.174 (0.029, 0.305)
Cumbria		112	5.89		15.78	0.44	0.62	0.287 (0.216, 0.358)
Scotland		94	5.47		37.14	0.47	0.60	0.220 (0.153, 0.295)
Cumbria Historic		28	4.38		28.00*	0.54	0.68	0.213 (0.050, 0.357)

Table 5.4. Pairwise Fst (below) and G'st (above) for sites sampled in Cumbria (A, n=112)) and Scotland (B, n=94)). Significance: * p<0.05; ** p<0.01; *** p<0.001.

A	Braithwaite (n=30)	Ennerdale (n=29)	Finglandrigg (n=30)	Middlesceugh (n=23)
Braithwaite	-	0.0204**	0.0441***	-0.0097
Ennerdale	0.0405**	-	0.0561***	0.1004**-*
Finglandrigg	0.0609***	0.0705***	-	0.1147***
Middlesceugh	0.0158	0.1186***	0.1257***	-

B	Islay (n=22)	Mull (n=12)	Oronsay (n=30)	Tayvallich (n=30)
Islay	-	0.0767**	0.0959**	0.1512***
Mull	0.1185**	-	0.0811**	0.0711***
Oronsay	0.0769***	0.1158**	-	0.0883***
Tayvallich	0.1495***	0.1033***	0.0924***	-

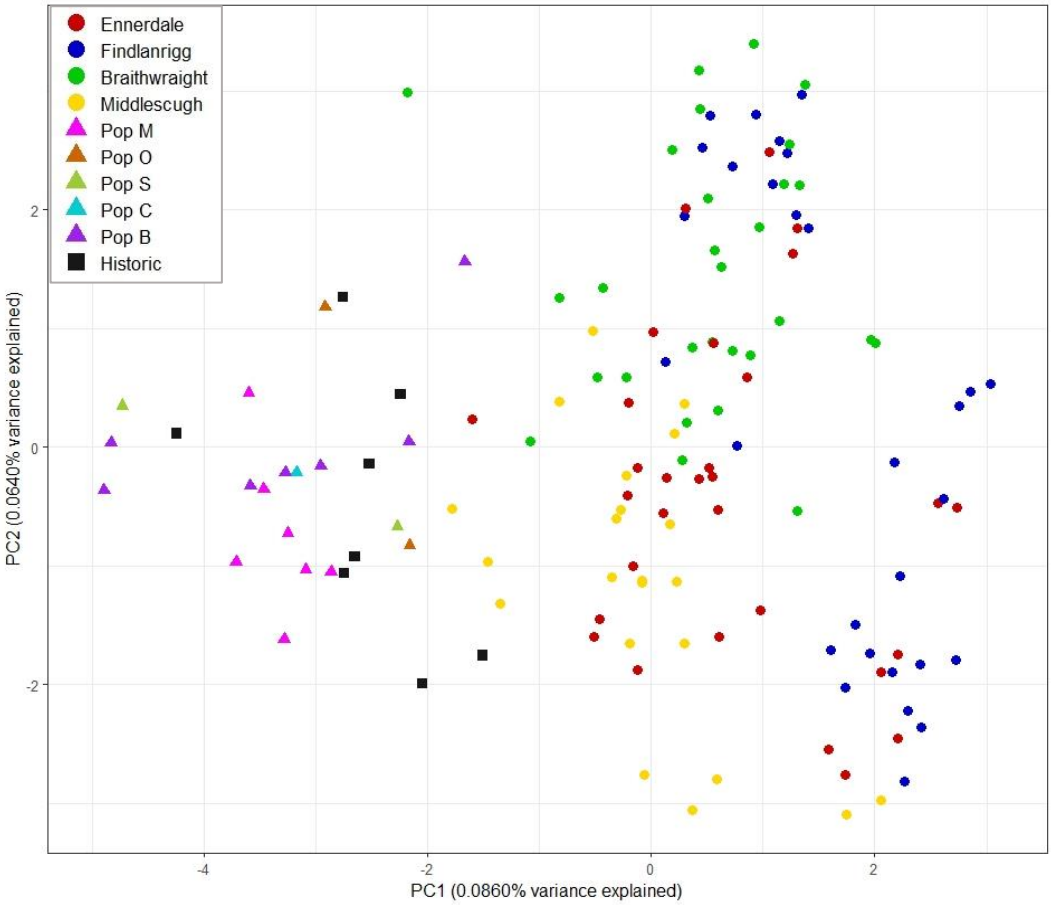

Figure 5.6. Principal component analysis for reintroduced sites (circles) and historical (1920-1930) specimens from Cumbria (squares). Samples collected in the 1970-1990s (triangle) act as a proxy for the Cumbrian founders of the captive breeding population as they are the most recent available specimens.

Within the Scottish sites, two (Tayvallich and Mull) were donors to the Cumbria captive breeding program and two (Islay and Oronsey) were not. Scottish donor and non-donor sites showed similar levels of diversity and variation. These populations are explored more fully in chapter 3. Pairwise Fst and G'st values between the Cumbrian population and the donor populations are lower than between Cumbria and non-donor populations (Table 5.5).

Table 5.5. Fst (A) and G'st (B) within populations and pairwise between populations. Donor sites (Tayvallich and Mull) are known to have provided founders for the Cumbrian captive breeding project, non-donor sites (Islay and Oronsey) did not. Significance: * p<0.05; ** p<0.01; *** p<0.001, N=206.

A	Cumbria (n=112)	Scotland donor (n=42)	Scotland non-donor (n=52)
Cumbria	0.0673***		
Scotland donor	0.2316***	0.1133***	
Scotland non-donor	0.3090***	0.0803***	0.0705**

B	Cumbria (n=112)	Scotland donor (n=42)	Scotland non-donor (n=52)
Cumbria	0.0654***		
Scotland donor	0.2224***	0.0919***	
Scotland non-donor	0.3210***	0.0686***	0.0904**

PCA comparison between the reintroduced population and the populations which represent the two parts of the founder population show that separation between all three groups is almost complete (Figure 5.7). The Scottish samples are separated completely from all Cumbrian material on PC1. The reintroduced population is distinct from the historic Cumbrian populations on the PC2 axis, with the exception of one point There is no separation between donor and non-donor sites in the Scottish clustering and both show comparable levels of genetic diversity.

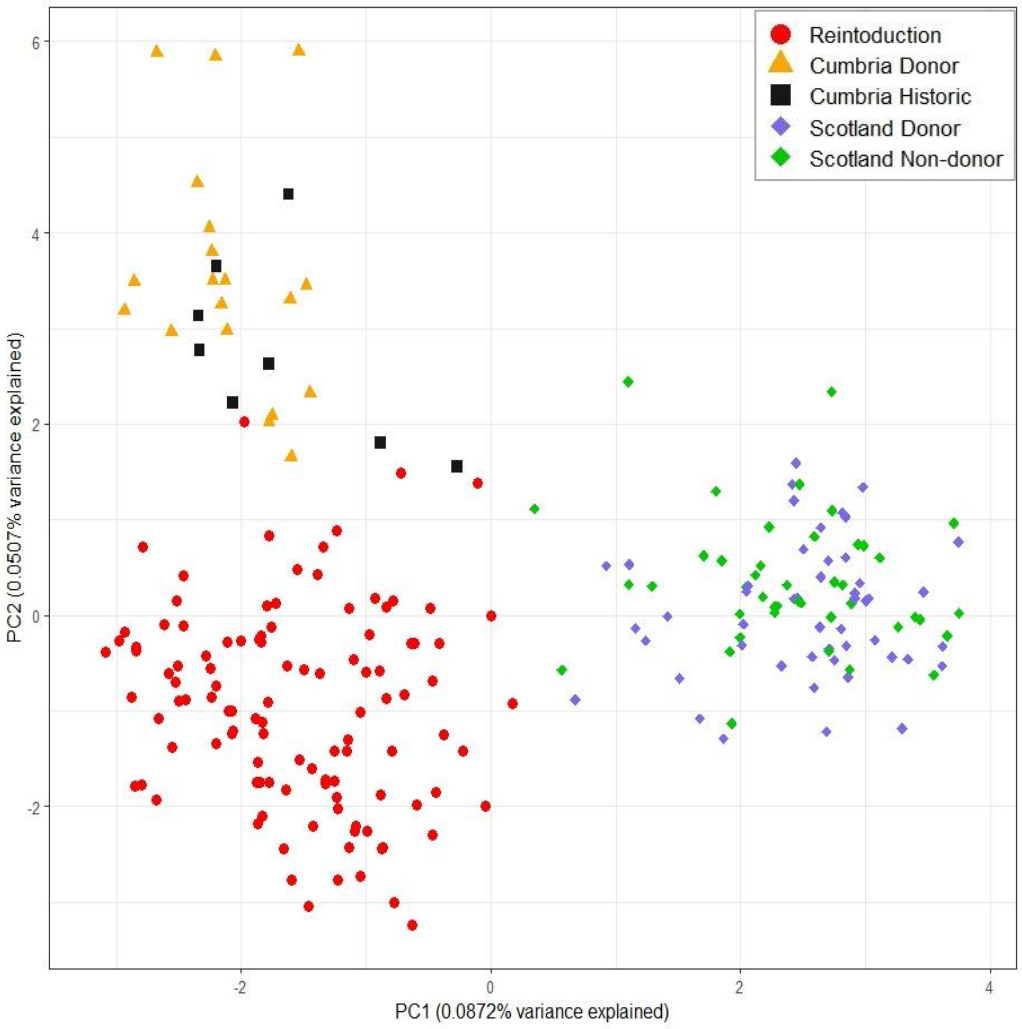

Figure 5.7. Principal component analysis of the reintroduced population in Cumbria and the two donor populations, as well as the historic Cumbrian samples. Scottish sites (diamonds) are distinguished between those which contributed to the captive breeding program (green) and those which did not (blue).

5.4 Discussion

At present all four of the original reintroduction sites display similar levels of genetic variation which are greater than the levels of variation displayed by the four Argyll sites included in this study, this is expected due to the diverse origins of the Scottish founders of the Cumbrian reintroduction which were collected from 19 sites. Pairwise Fst and G'st comparison between Cumbria sites (Table 5.4) shows significant levels of differentiation between the sites (0.0158–0.1257 and -0.0097–0.1147, respectively)

similar to those seen in Argyll (0.0769–0.1495 and 0.0711–0.1512, respectably) and mostly within the range of those seen in Ireland (0.0293–0.1671 and 0.0243–0.1615, respectively, Chapter 2).

Populations of the Marsh Fritillary naturally go through cycles of expansion and reduction, as was first reported by Ford & Ford (1930), monitoring by the Cumbrian Marsh Fritillary Action Group (CMFAG) shows this cycle has not yet occurred in Cumbria (unpublished data) and critically the extinction/recolonization dynamic of a metapopulation has not yet occurred at the four reintroduction sites as they have been continuously occupied and are too geographically separated for migration between the populations to be likely. This may well account for the higher allelic richness seen at the Cumbrian sites compared with those in Scotland and Ireland, the Cumbrian population has not undergone a bottleneck since its founding, and therefore has not experienced the loss of alleles seen in the Irish and Scottish populations which will have undergone such bottleneck cycles repeatedly.

Evidence for the occurrence of bottlenecks in the historic Cumbrian population can be found in the lack of private alleles in the later (donor) population. Approximately 10% of the alleles found in the 1920-1930 populations are not found in any other population, including the 1970-1990 population, their loss probably being due to one or more bottlenecks in the intervening period. Studies which compare historic and current levels of genetic diversity are rare, likely due to the challenges of working with ancient DNA (Nicholls, 2005; Bi *et al.*, 2013; Burrell *et al.*, 2015). However a similar loss of genetic diversity between historic and present populations has been documented in the African lion (*Panthera leo*) in the Kavango-Zambezi conservation area (Dures *et al.*, 2019). The modern population is 12-17% smaller than the historic populations in the late-19th and early-20th century and contains 15% lower allelic diversity. The historic population also contained what are termed “ghost alleles” which

are not present in the modern population, similar to what is seen when comparing the 1920s Cumbrian Marsh Fritillary with later populations.

The occurrence of private alleles in the reintroduced Cumbrian populations which were not recorded in the Argyll sites is an interesting finding. This may be due to one or more of three factors; such alleles are the result of mutation in the markers, the alleles are currently at low frequency in Argyll and therefore were not detected, or the alleles are unique to Cumbria and demonstrate the persistence of the original Cumbrian population, either in the form of alleles preserved in the captive population or in previous undetected wild populations that have interbred with the reintroduced populations.

The possibility of the persistence of a previously undetected Cumbrian population remains, since the absence of evidence is not evidence of absence, however it is highly unlikely due to the close monitoring of the species in the region over the decades that it took to decline to extinction (Porter & Ellis, 2011). There are also no reported occurrences of the species outside of the reintroduction sites or areas close enough to those sites that the specimens seen there are very likely the result of natural colonisation and the beginnings of natural metapopulation formation (CMFAG, pers. comm.).

Although the persistence of a small undetected Cumbrian population cannot be completely eliminated, it is unlikely bordering on impossible that sufficient persisted undetected to account for the 15.78% private alleles observed. Nor is it possible that these are entirely due to the contribution of the Cumbrian founders to the captive breeding, as these are all suspected to be the offspring of a single pair (Porter & Ellis, 2011) and thus at most only a few alleles per locus could have been contributed. Given that the populations had been declining for a number of years it is likely that the actual number of unique alleles contributed by the Cumbrian founders was significantly less due to the effects of inbreeding.

It is likely then that the cause of the high number of private alleles in the reintroduced population, and the observed separation between the Argyll and reintroduced populations on the PCA is the result of the composition of the Argyll founders of the captive breeding population. The Argyll component was collected from 19 donor sites of which this study revisited only two, resulting in ~90% of the genetic variation from the Argyll founders not being represented in this analysis. Despite this, pairwise comparison has shown that Cumbrian specimens are more genetically similar to the Scottish donor populations than to the non-donor populations, though this was not reflected in the PCA analysis. This suggests that the Argyll dataset is at least partly representative of the founders used for the reintroduction.

In the nine generations since the reintroduction was carried out some degree of genetic differentiation as evolved between most of the sites as demonstrated by the pairwise Fst and G'st scores. Middlesceugh is of particular interest as it not only has the only non-significant pairwise score with Braithwaite, but also the highest pairwise scores with Ennerdale and Finglandrigg. As these microsatellite markers had previously been found to be selectively neutral (Smee *et al*., 2013) adaption or selection pressures are unlikely. Moreover as the populations have only been separate for nine generations it is unlikely that there has been sufficient time for sufficient mutations to occur to account for this differentiation (Ellegren, 2000). Therefore, the most probable cause is genetic drift. Genetic drift has the greatest effect in small populations (Willi *et al*., 2007; Whitlock, 2010), this explains the pattern seen for Middlesceugh, in three years following the initial reintroduction Middlesceugh had a significantly lower web count (the accepted way to survey for the Marsh Fritillary is to count larval webs) compared to the other three sites (Figure 5.8) (Porter & Ellis, 2011) and this has continued to be the case in subsequent years (CMFAG, pers. comm.). The lack of differentiation between Middlesceugh and Braithwaite is therefore

due to chance and such differentiation may reasonably be expected to occur in the future.

The reintroduction had two stated goals; to re-establish the Marsh Fritillary in Cumbria and to preserve Cumbrian specific genetic material (Doyle, 2008). With respect to the first goal the project was certainly a success in that the Marsh Fritillary is present as a self-sustaining population in Cumbria (CMFAG, pers. comm.), however with respect to the second goal the result is somewhat less clear.

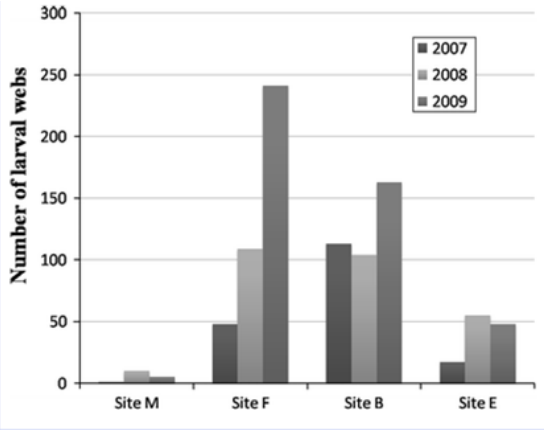

Figure 5.8. Larval web counts for the first three years following reintroduction. From Porter & Ellis (2011).

The two founder populations contributed a disproportionate number of alleles, potentially a maximum of 190 alleles per locus for the Scottish founders compared to 4 per locus for the Cumbrian founders and given the long decline and attendant bottleneck of the Cumbrian population it is likely to be less than this. In such a situation genetic swamping, the total replacement of the local genotypes with incoming genotypes, is to be expected (Lenormand, 2002) and is a concern with some reintroduced populations (Veale & Russello, 2016). However, this does not seem to have occurred in the Cumbrian reintroduction. The clear separation of the reintroduced and Scottish populations in the PCA offers possible evidence of the persistence of Cumbrian specific alleles as does the very slight overlap, on the PC2 axis, between the historic Cumbrian and reintroduced populations.

There have been very few studies of the genetic differentiation between sites after a reintroduction, however similar patterns of population differentiation, though to a greater extent, have been seen in the reintroduced population of the Lage Blue butterfly (*P. arion*) in the UK. In this case all UK populations are reintroduced and have similar levels of genetic diversity but differing allele frequencies (Andersen *et al*., 2014). The Large Blue reintroduction took place over two decades before the study accounting for the greater extent of the population differentiation, a study on two locally reintroduced *Maculinea* spp in Netherlands found no significant population differentiation after five generations (Wynhoff, 2001).

Similar to the results presented here, the study by Brekke *et al*. (2011) on translocated and source populations of the hihi (*Notiomystis cincta*), a New Zealand endemic bird, found that while high levels of genetic diversity were maintained in the translocated populations compared to the source there was also a significant level of divergence between source and translocated populations as well as between individual translocated populations. The similar levels of genetic diversity between natural and reintroduced populations of Marsh Fritillary is similar to what is seen in the Griffon vulture (*Gyps fulvus*) where similar levels of genetic variation have been found in natural and reintroduced populations throughout Europe (Le Gouar *et al.*, 2008).

The late stage of the decline at which the genetic management and supplementation, in the form of captive breeding, was implemented is certainly the reason for the loss of the many Cumbrian specific alleles which were present in the 1980s samples. Had the captive breeding project been implemented ten to twenty years earlier, when there was still more than a single population remaining, then the result would have likely been a more balanced contribution from the two founder populations.

Based on the findings of this study it is recommend that no further genetic management (e.g. translocations or populations supplementations) of the Cumbrian population is undertaken at this time, none of the reintroduced populations studied are at risk of inbreeding depression and as new colonies are establishing naturally it is reasonable to assume that gene flow between the reintroduced populations will also naturally establish with time as has been seen following reintroductions in other species (Le Gouar *et al.*, 2008).

On a broader scale, it is recommended that the managers of other declining populations of the Marsh Fritillary strongly consider adopting this management strategy, and if necessary the captive breeding methodology, used in Cumbria (for details see Porter & Ellis, 2011). Where a population is not in immediate danger of extinction, captive breeding may be unnecessary, and a similar genetic rescue could be carried out by releasing late stage instar, collected from a suitable donor population, at the site which is either known or suspected to have low levels of genetic diversity. A low level of gene flow, represented by a few individuals from external sources breeding successfully each year, is sufficient to avoid inbreeding depression while still retaining any locally advantageous adaptations (Åkesson *et al*., 2016; Gustafson *et al.*, 2017). This strategy, including the genetic rescue, should also be considered for other Lepidoptera species which have similar life histories and conservation concerns (Frankham, 2015; Ralls *et al.*, 2018).

6 The butterfly effect: What insights can the Marsh Fritillary provide on wider conservation questions?

6.1 General Discussion

6.1.1 Sourcing founders for reintroduction

The earliest reintroduction is believed to have been carried out in 1907 with the release of 15 American bison (*Bison bison*) into a reserve in Oklahoma (Kleiman, 1989). Many of the early reintroductions were a matter of releasing animals into a site and hoping for the best, unsurprisingly many failed to establish viable populations (Seddon *et al.*, 2007). In Lepidoptera it was not uncommon for collectors and breeders to simply release excess stock into the wild, with little to no consideration given to the ability of the habitat to support the species, a practice which rarely resulted in a successful establishment (Oates & Warren, 1990). Modern practice is guided by the theoretical framework provided by the guidelines of the IUCN Species Survival Commission (IUCN/SSC). These guidelines recognise the importance of sourcing suitable founders for reintroduction programs (IUCN/SSC, 2013).

Broadly, founder selection fits into two categories; selecting founders which are pre-adapted to the reintroduction site by matching source to destination (either genetically or environmentally) and incorporating as much variation as possible and allowing adaptation of founders to environment to take place *in-situ* at the reintroduction site (Houde *et al*., 2015). The IUCN guidelines favour the matching of source to destination while recognising that such decisions must be made on a case by case basis and that sometimes more radical techniques must be employed (IUCN/SSC, 2013).

Sourcing from a wide variety of populations with distinct genetic and/or environmental backgrounds may be referred to as the adaptive potential strategy (Houde *et al*., 2015). This provides high levels of genetic variation which can be

beneficial to the establishment and persistence of the reintroduced populations, however there is also a risk of genetic or behavioural incompatibility and outbreeding depression (IUCN/SSC, 2013). Such a case was documented in the fresh water fish *Cottus cognatus* following a mixed-source reintroduction; hybrid offspring in the second generation had reduced growth compared to pure strain offspring, suspected to be due to the disruption of co-adapted gene complexes (Huff *et al.*, 2011). However in *Jacquemontia reclinata* (a coastal perennial vine endemic to south-eastern Florida) it was found that individuals of mixed ancestry had better survival and resistance to stochastic disturbance than did individuals from a single source (Maschinski *et al.*, 2013).

Sourcing based on matching source to destination may be referred to as the pre-existing adaptation strategy (Houde *et al.*, 2015). When matching based on genetic similarity or shared ancestry it is common to use neutral markers such as microsatellites or SNPs, sometimes combined with functional markers. Matching based on the environmental traits of the source and reintroduction sites can also be supplemented with examination of functional markers or quantitative traits, assuming individuals from the proposed reintroduction site still exist, either alive or preserved. For both ancestral and environmental matching geographical distance may be used as a proxy, however this may not always be suitable (Lawrence & Kaye, 2011). Source matching based on physiological characteristics was used in the selection of the donor population for the reintroduction of the Large Blue butterfly (*P. arion*) to the UK, in this case the decision to use the population in Öland, Sweden, as the source was also influenced by the size of the proposed donor populations, with it being the largest surviving of the northern populations in Europe (Andersen et al., 2014). For the Large Copper (*Lycaena dispar*), incorrect matching of source and destination is the ascribed cause of failure for the 1909 reintroduction attempt, this tried to replace

the native univoltine subspecies *L. d. dispar* with the bivoltine subspecies *L. d. rutilus* (Asher *et al.*, 2001).

The reintroduction of the Marsh Fritillary to Cumbria incorporated both of the strategies described above, though it was ancestry matching that was the primary consideration as the intention was to reintroduce to multiple sites and thus pure environmental matching was unsuitable. Concerns about the genetic diversity of the remaining Cumbrian individuals, the larvae collected from the wild were believed to all be full-siblings, led to the selection of additional founders for the captive breeding program (Porter & Ellis, 2011). The use of Argyll as the source region was guided by Joyce (2001) which grouped Cumbria with Scotland as part of the Northern metapopulation, however the decision to take five larvae from nineteen different sites rather than a greater number of larvae from fewer sites was guided by a desire to maximise the genetic diversity of the captive population (Porter & Ellis, 2011). It also reduced the risk that removing founders might have presented to any one source population in line with the IUCN/SSC guidelines.

The success of the Cumbrian reintroduction shows that the sourcing of founders can be a combination of the strategies described by Houde *et al.*, (2015). Previous work did not find any confirmation of the benefits of the adaptive potential strategy, while both ancestry and environmental matching increases the likelihood of the reintroduction succeeding (Houde *et al.*, 2015). However, this does not mean that adaptive potential should be discarded completely. Using ancestry matching to determine a source region or selection of sites and then sampling broadly incorporates both strategies and has been demonstrated to be successful with the Marsh Fritillary in Cumbria which is presently extant at 18 sites, four of them natural colonisations (Porter, pers. comm.). It also has the added advantage of reducing the pressure on any one source population. This is particularly important when working with endangered species under legal protection.

6.1.2 Neutral and adaptive measures of variation

The use of Fst and similar statistics to assess the differentiation between populations has a long history. These measures are based on neutral markers such as microsatellites and this has led to concerns that the use of them might fail to capture quantitative genetic variation, that is variation which is under selective pressure. For this reason Qst was developed as an analogous measure of differentiation for quantitative traits (Spitze, 1993).

Comparison of Fst and Qst values has been used to provide information about the selection pressures on different populations. Where Qst>Fst it is interpreted as differential directional selection on the quantitative trait between populations while Qst<Fst is interpreted as selection favouring the same phenotype in each population. In cases where Qst=Fst the variation in the quantitative trait is no different than would be expected by genetic drift alone (Allendorf *et al.*, 2013). However care must be taken in the interpretation of such results as a review of empirical studies has shown that the relatively high mutation rates of neutral markers may bias comparisons (Edelaar *et al.*, 2011). There is also evidence that some of the effects may be species specific and that the selection of quantitative traits may be introducing bias into the analysis (Miller et al., 2008). Finally it must be noted that some statistics used as an alternative to Fst, such as G'st, are not valid for comparison with Qst due to their underlying assumptions and structuring (Edelaar & Bjõrklund, 2011).

The use of Qst in conservation biology has differed between plants and animals. For plant species common garden experiments, often used to measure or estimate Qst, have long been used to identify suitable seed sources and transfer zones to limit maladaptive risks such as outbreeding depression (Hufford & Mazer, 2003). A common garden experiment is suggested as a method to determine the genetic basis of resistance to white pine blister rust in whitebark pine (*Pinus albicaulis*) and limber pine (*P. flexilis*) in North America, this information could then be used to

inform selection of suitable resistant stock to introduce into infected areas in the United States (Schoettle & Sniezko, 2007).

Common garden experiments are not feasible for animal species and estimates of Qst in the wild can be problematic (Allendorf *et al*., 2013). Given these difficulties phenotypic variation (Pst) is sometimes used as a substitute or proxy for Qst (Sæther *et al*., 2007). However extreme care must be taken when interpreting Pst due to concerns regarding the biasing effect of phenotypic plasticity responding to environmental conditions during the development of the organism (Pujol *et al.*, 2008) and comparisons of Fst-Pst should be conservatively interpreted (Brommer, 2011). Nevertheless Qst-Fst comparisons have been used to examine ecotype divergence in a number of animal species (Manier *et al.*, 2007; Eroukhmanoff *et al.*, 2009).

Use of Qst to inform a reintroduction program has been little documented in the literature, possibly due to a lack of knowledge of the genetic control of various adaptive traits in many species in need of reintroduction programs. When a quantifiable change has led to the need for reintroduction, as with the appearance of white pine blister rust discussed above, Qst can be very beneficial for finding suitable genotypes, for example those which are resistant to the disease (Schoettle & Sniezko, 2007).

In cases where there are likely to be multiple adaptive traits which are beneficial or Qst is otherwise impractical then Pst may be used as a proxy (Sæther *et al.,* 2007) which in turn leads to its own set of practical problems. These primarily will come from obtaining phenotypic measurements outside of a captive environment (Storfer, 1996). Obtaining phenotypic measurements for the original population at the reintroduction site may be difficult or impossible depending on how long ago the species became extinct at the site. With small and/or highly isolated populations, as is often the case with endangered species, phenotypic traits can become fixed in the

population which may be either maladaptive or indicative of inbreeding depression (Roelke *et al.*, 1993; Johnson *et al.*, 2010)

Museums can serve as a reference collection however there will be limitations in terms of the number of specimens and the original source of those specimens. The Marsh Fritillary is an example of a species which has been widely, though not evenly, collected. Personal observation based on records from museum collections has demonstrated that certain regions of the British Isles are heavily over represented. As an example, hundreds of specimens from Hod Hill in Dorset are available in various collections while only three specimens are available from the entire county of Yorkshire (an area of over 11,000km^2) (unpublished data). In addition, museum specimens cannot provide information on behavioural traits.

Due to the limitations and challenges associated with the use of Qst or Pst in real world situations it is unlikely that their use will be widely adopted in the near future or that they will replace Fst and associated measures of neutral diversity in the selection of reintroduction founders outside of specific scenarios such as a desire for disease resistance or environmental tolerance. However, disregarding adaptive variation completely would be unwise and has been known to lead to the failure of reintroductions (Asher *et al.,* 2001).

6.1.3 Reintroduction failures

Defining success and failure for reintroductions is not as simple as it first may appear, the criteria for failure, the absence of the species from an area, is clear but how long must a species be present for a reintroduction to be defined as a success? The reintroduction of the Large Blue butterfly (*Phengaris arion*) in the UK and the Oregon silverspot butterfly (*Speyeria zerene hippolyta*) are both defined as successful in the IUCN Global Reintroduction Perspectives case studies despite the fact that the latter

reintroduction had only taken place the previous year (Soorae, 2018) while the former has been established for decades (Soorae, 2011). This problem of definition has been discussed in the literature however there appears to be no consensus on how to solve it (Robert *et al.*, 2015).

The success or failure of reintroduction attempts are rarely documented in the primary literature (Daniels *et al.*, 2018). For Lepidoptera, reintroductions have, historically, been primarily carried out by amateurs, with little to no documentation in even the grey literature as to what was done and if it was successful (Oates & Warren, 1990). More recently, when reintroductions have been fully documented in the literature they are generally successful (e.g. Witkowski *et al.*, 1997; Porter & Ellis, 2011). The six editions of the IUCN Global Reintroduction Perspectives include five butterfly case studies, of these only one is deemed a failure. Indeed, the publications do appear to suffer from some level of publication bias as of the 384 case studies with known outcomes, only 16 (4.17%) are deemed to be failures (82 highly successful (21.35%), 149 successful (38.80%) and 137 partially successful (35.68%)). This bias has been noted elsewhere along with a higher incidence of publication of mammal and bird reintroductions though how much this may in part reflect a bias in reintroductions carried out is unclear (Bajomi *et al.*, 2010).

The failure of the Miami Blue butterfly (*Cyclargus thomasi bethunebakeri*) to establish prolonged populations in any location was attributed to stakeholder conflicts limiting the options for reintroduction sites, combined with difficulties in producing sufficient larvae and stochastic disruption in the form of a tropical cyclone at the reintroduction sites (Soorae, 2010). There are other accounts of failed reintroductions such as the Black-veined White butterfly (*Aporia crataegi*) which was introduced from Spain to the UK in 1974 but only established temporary colonies before becoming extinct once again (Asher *et al.*, 2001). The cause is unclear but it is suggested that

Britain, being on the edge of the species range, was too extreme an environment for the transplanted butterflies to persist (New, 1997b).

Significant attempts have also been made to reintroduce the Large Copper butterfly (*Lycaena dispar*) to the UK; due to the extinction of the UK subspecies *L. d. dispar* alternative subspecies from continental Europe have been used when attempting reintroductions. The first of these occurred in 1909 but failed quickly, this was due to the selection of an inappropriate subspecies, *L. d. rutilus,* which is bivoltine when the native subspecies had been univoltine (Asher *et al.*, 2001) creating an ecological mismatch between the species phenology and the environment. Later attempts were undertaken from 1927 onwards to introduce a univoltine subspecies *L. d. batavus*. Some colonies persisted until the 1990s however they were never truly self-sustaining and required periodic release of captive bred stock and the protective netting of larvae in the field (Asher *et al.*, 2001). This failure to fully establish and the ultimate extinction of the species in the UK is attributed in part to the very limited availability of habitat, and the isolation and dryness of the remaining fenland habitat (New, 1997b; Asher *et al.*, 2001). Fenland drainage alters habitat and is also indicated as a cause of the failure of the swallowtail *Papilio machaon* to establish in Wicken Fen (Dempster & Hall, 1980).

Examination of the IUCN reintroduction case studies reveals that insufficient, incorrect or poor quality habitat is a frequent cause of reintroduction failures, along with limited knowledge of the ecology or natural history of the species to be reintroduced (Soorae, 2008, 2010, 2013, 2016, 2018). Along with lower quality habitat, a lack of refugia from predation is believed to have been part of the reason that the brown treecreeper (*Climacteris picumnus*) failed to establish in south-eastern Australia. Predation was also one of the reasons suggested for the failure of the Black-veined White butterfly reintroduction to establish in the UK (New, 1997b).

The success of the Cumbrian reintroduction in establishing self-sustaining and expanding populations of the Marsh Fritillary can be attributed to the close partnership between volunteers and landowners, who were in some cases one and the same (CMFAG, pers. comm.). Habitat management and preparation prior to the reintroduction, including the planting of thousands of larval food plants at the sites, also assisted in the establishment of reintroduced populations (Porter & Ellis, 2011). In this CMFAG had learnt from the failed reintroductions carried out in 1997 and 1998 which are believed to have failed in part due to a lack of management and preparation undertaken at the release sites.

6.1.4 Habitat loss and fragmentation

Although theoretically habitat fragmentation can occur without habitat loss (Figure 6.1), it is hard to conceive of a practical scenario resulting from anthropogenic action where this would be the case, except for the creation of new habitat as part of conservation mitigation (Franklin *et al*., 2002). Fragmentation therefore has two key components: habitat loss and insularization (Wilcox & Murphy, 1985). To this some would add a third component; increased edge effect (Franklin *et al*., 2002; Wilson *et al*., 2016).

The relative importance of habitat loss and habitat fragmentation can be difficult to quantify. Statistical modelling suggests that the amount of habitat accounts for 68% of the variance in population size compared to just 13% accounted for by fragmentation and that the effects of fragmentation are equivalent to the loss of 15% of good quality habitat (Wiegand *et al.*, 2005). However modelling also suggests that while a few large populations may result in the maximum carrying capacity, a greater number of medium sized populations results in the maximum persistence time for a species (Ovaskainen, 2002).

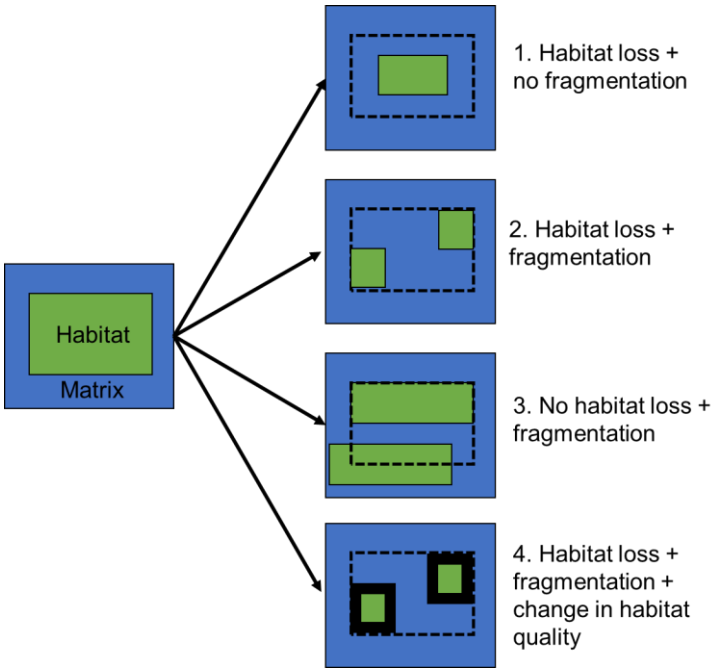

Figure 6.1. Four cases illustrating the relationship between habitat loss and fragmentation. Redrawn from Franklin *et al.* (2002).

Confounding factors such as the trophic level and degree of specialism may be masking the effects of fragmentation (Ewers & Didham, 2006). Many studies do not adequately take into account or control for the confounding effect of these variables (Smith *et al.,* 2009). Nor do they necessarily take into account the response time of individual species and that the continued occurrence of a species in a fragmented habitat may be an extinction debt (Ewers & Didham, 2006).

The effect of habitat loss is simple to predict, less habitat can support less individuals, however understanding the response of an individual species to habitat fragmentation requires an in-depth understanding of the requirements and ecology of the species (Wiegand *et al.*, 2005) such that even applying general rules may not be possible in certain situations such as metapopulation species (Ovaskainen, 2002). It may very well be that “the uniqueness of species and the landscapes in which they live confound simple analysis.” (Wiegand *et al.*, 2005, p. 108).

6.1.5 Habitat fragmentation and genetic diversity.

Separating out the impacts of the two aspects of habitat fragmentation discussed by Wilcox & Murphy (1985) is rarely done. One such example comes from the creation of Qiandoao Lake in Zhejiang Province, China, which created over a thousand forested islands of various sizes. As islands in a freshwater lake, the exact size of each habitat fragment is known, as is the date at which they were created (beginning 1959 with the completion of a hydroelectric dam). The genetic diversity of *Castanopsis sclerophylla*, an evergreen tree species, was investigated across the islands. Analysis concluded that the loss of habitat associated with the flooding of the area was responsible for an initial loss of genetic diversity while insularization was responsible for the later loss of genetic diversity as well as the population differentiation and inbreeding levels observed on different islands (Zhang *et al.*, 2012).

There have been other studies that suggest habitat fragmentation (in the broad sense) does not appear to affect some species (Zartman *et al.*, 2006; Otálora *et al.*, 2011; Matesanz *et al*., 2017) and that not all species will respond to the same extent, notably plant species capable of selfing respond less quickly to the effects of fragmentation than obligate outbreeding species (Honnay & Jacquemyn, 2007). Even when species are theoretically expected to respond in a certain way to fragmentation some confound these expectations by responding in an unpredictable manner, this suggests that the underlying processes are still not well understood (Keyghobadi, 2007).

Nevertheless, habitat fragmentation does appear to affect many species. For self-incompatible plant species such as *Linnaea borealis* fixation of alleles due to fragmentation has resulted in only 16% of fragments in a Scottish National Park being capable of producing seeds (Wiberg *et al.*, 2016). Furthermore, in long lived plant species such as trees there is the possibility of a genetic extinction debt (Fuller & Doyle, 2018). In this the effects of habitat fragmentation can be masked as the adult

trees, often the ones which are sampled, show the relic genetic diversity of the historically less fragmented landscape while the offspring (often unsampled) show the reduced genetic diversity of the presently fragmented landscape (Vranckx *et al.*, 2012).

The conservation genetics fragmentation literature is heavily skewed in favour of plants (Schlaepfer *et al.*, 2018). However temporal delay in the effects of fragmentation on genetic diversity have also been noted in animal species. The level of genetic diversity observed in the butterfly *Parnassius smintheus* in the Canadian Rocky Mountains correlated better with the historic forest cover patterns than with the present while the reverse is true for the population differentiation which correlates much closer with modern forest cover (Keyghobadi *et al.*, 2005).

Loss of genetic diversity associated with habitat fragmentation has been observed in many other species (Dixon *et al.*, 2007; Mhemmed *et al.*, 2008; Bruggeman *et al.*, 2010; Wang *et al*., 2017; Barmentlo *et al.*, 2018; Wan *et al*., 2018). However, it is also clear that the effects of fragmentation can take time to manifest (Benedick *et al.*, 2007) and that although older fragments generally have lower genetic variation (Rivera-Ortíz *et al.*, 2015), the process of genetic erosion is not necessarily a linear relationship (Pflüger *et al.*, 2019). Analysis has also shown that the degree to which a species is affected, or the time scale over which it occurs, is species specific and is influenced by ecological and life history traits (Honnay & Jacquemyn, 2007; Rivera-Ortíz *et al.*, 2015; Lino *et al.*, 2019).

Given this, it is important that appropriate management and conservation action is initiated as soon as possible following fragmentation in order to minimise any effects of the fragmentation. Moreover, it is important that this management is species specific and guided by what is known of the ecology and genetics of the species.

6.2 Conservation implications and management recommendations for the Marsh Fritillary.

In the UK the Marsh Fritillary is legally protected from being intentionally (and in Scotland recklessly) killed, injured, disturbed or taken from the wild under the Wildlife and Countryside Act 1981 and the Northern Ireland Wildlife Order 1985. The sale of the Marsh Fritillary, in whole or in part, is also prohibited by the same laws. Although this law has never led to prosecution for the Marsh Fritillary, there has been at least one conviction under this Act for the taking and killing of the Large Blue butterfly (*Phengaris arion*) (Butterfly Conservation, 2017b). This level of legal protection, and willingness to enforce it, is beneficial to the long-term survival of the species and similar laws should be adopted in other parts of the species range. The Wildlife and Countryside Act also makes release of the Marsh Fritillary without a licence an offence, this helps to reduce the possibility of negative genetic effects, such as inbreeding or outbreeding depression, resulting from an ill planned reintroduction.

Legal protection is also afforded to the Marsh Fritillary via a Special Area of Conservation (SAC) in Cumbria (SAC code UK0030126). However this site was designated in 2005 (English Nature [now Natural England], 2005) which is prior to the extinction and reintroduction documented in Porter & Ellis (2011). Today the SAC protects only the weakest of the four original reintroduction sites. While this is beneficial it would be better for the conservation of the species in the region if the designation was shifted either to a site which contains a greater percentage of the total population in the region or, ideally, expanded to include multiple sites. In this situation the minimum would be to expand it to include the other three original reintroduction sites which are beginning to show genetic differentiation from each other (Chapter 5). As these have been established the longest these are most likely to be at the centre of naturally establishing metapopulations, which was one of the aims of the reintroduction (CMFAG, pers. comm.).

Given the lack of recent taxonomic treatment of the Marsh Fritillary and any possible subspecies that may be present in the British Isles, it would be inadvisable to consider any translocations to or from the region until the work by Korb *et al.* (2016) has been expanded to include specimens from the British Isles. Given the evidence of Whitla (2019), that Ireland may have been colonised by a single event, combined with the historic view that there is an Irish subspecies (Birchall, 1873; Kane, 1893; Ford, 1945; Kloet, 1972), it would further be inadvisable to move individuals between the British and Irish landmasses. Also, as *Euphydryas aurinia* ssp/f. *hibernica* is reported from Scotland, movement between the northern and southern regions identified by Joyce & Pullin (2001) would not be recommended until further taxonomic work has taken place. Although at the time of the work by Joyce and Pullin, Cumbria was considered to be somewhat distinct from Scotland this was prior to the reintroduction and today the Cumbria population should be considered to be predominantly Scottish by descent in terms of genetic composition and origin (Porter & Ellis, 2011; Chapter 5)). The exception to this would be if the intention was reintroduction to an intermediate geographical region of the country or an area from which the Marsh Fritillary had been absent for a number of decades, such as Yorkshire or the Midlands, and the maximum amount of genetic diversity was sought with the intention to allow adaptation to conditions to occur *in-situ*.

Following the direction of the UK Post-2010 Biodiversity Framework (which succeeds the UK Biodiversity Action Plan, BAP) to “improve the status of biodiversity by safeguarding ecosystems, species and genetic diversity” (JNCC & Defra, 2012, p. 6), the primary aims of conservation efforts are to maintain the Marsh Fritillary in the landscape, re-enforce existing population networks (metapopulations) and restore it to areas from which it has been lost. The chances of survival of individual populations can be strengthened by the creation of additional habitat patches, within the ~400m distance identified in Chapter 4, to create a network of patches which together function

as a single population. The size of individual patches is less significant when multiple patches are functioning as a single population due to the high rate of inter-patch interaction in a patchy population (Aycrigg & Garton, 2014). This will increase the resilience of individual populations against catastrophes such as flooding, which is predicted to increase in occurrence and severity with the effects of climate change (Watts *et al.*, 2015).

Pending further confirmatory work, it may reasonably be assumed that a generational stepping-stone dispersal pattern occurs in the Marsh Fritillary (Chapter 3), this highlights the importance of reducing fragmentation in established Marsh Fritillary metapopulations. To this end, creating additional habitats between existing populations would allow for the establishment of additional populations to aid connectivity. These populations would best be spread across multiple habitat patches as described above (Figure 6.2).

The creation of a more robust network of sites around existing populations is part of the policy of Butterfly Conservation in Wales (Butterfly Conservation, n.d.) This organisation is currently raising funds to support the training of land managers and volunteers in identifying and monitoring the Marsh Fritillary as well as creating suitable habitat for it. The creation of additional habitat patches ~4km from presently established populations would be beneficial, these are at the low end of the range where patches function as independent populations (Chapter 4) but within the dispersal ability of the Marsh Fritillary identified in this thesis as well as by others (Table 1.1) so that the habitat could be naturally colonised if this was preferable to anthropogenic translocation to the site.

When seeking to reintroduce the Marsh Fritillary to an area where it has become extinct, or considering introducing it to an area where it had not previously lived but which is now suitable due to climate change or similar, adopting a variation

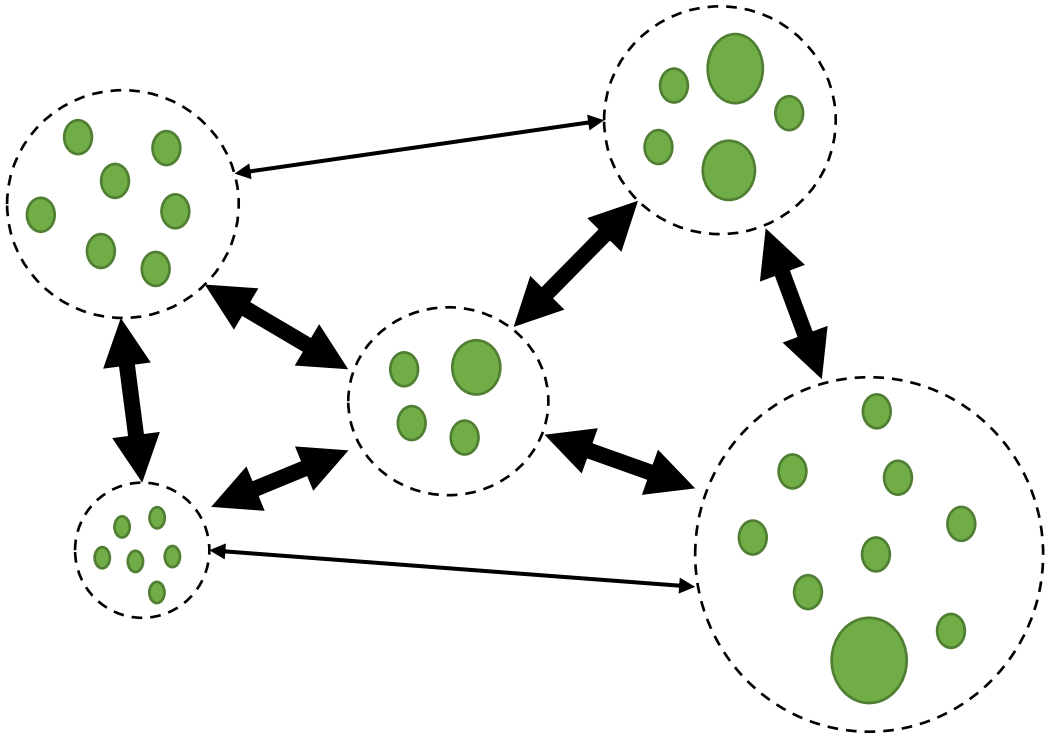

Figure 6.2. A model for a Marsh Fritillary metapopulation which can be used as a guide for a reintroduction program or modified to reinforce an existing population. Green circles are individual habitat patches and dotted lines define the areas functioning as a population within the metapopulation. Arrows show the level of movement, and thus gene flow, between sites with the thickness of the arrow indicating the amount of movement. Note that although gene flow between more distant populations does occur it is less frequent than between patches which are closer together.

of the approach taken in Porter & Ellis (2011) is advisable. This approach combined sourcing founders from sites with shared ancestry and selecting founders to maximise genetic diversity (shared ancestry was suspected with Scottish populations; a large number of sites were used as sources to maximise potential genetic diversity). This is demonstrated (Chapter 5) to produce a population that has genetic diversity comparable with a stable and healthy natural population. Where it is not possible to identify likely shared genetic ancestry then a similarity of environment between donor and reintroduction site should be sought, while also sourcing from as many sites as is possible.

A reintroduction of the Marsh Fritillary to Yorkshire is currently being considered, it has not been recorded in the county for over a century (Horsfall, pers. comm) and the last specimen known to be collected was in 1889 (NHM, 2014). Due to the lack of knowledge of the original population in the area it is recommended that founders are broadly sourced from across Scotland and those sites in Cumbria which are able to support the removal of larvae. Furthermore, it is recommended that a limited number of generations of captive breeding be used to build up a large number of individuals for use in the reintroduction. This large release number is believed to have been an aspect of the success of the Cumbrian reintroduction while not having any negative impact on the genetic diversity of the populations (Porter & Ellis, 2011; Chapter 5). In addition, where reintroductions continue over a number of years, the regular refreshing of the captive population with larvae sourced from established reintroduced sites is recommended. This was carried out in the Cumbrian reintroduction with no evidence of ill effect to either the donor reintroduced population or subsequently reintroduced populations (CMFAG, pers. comm.), it would reduce the risk of adaptation to captivity which is considered to have been an issue with the continued reinforcement of the Large Copper populations (New, 1997b).

All recommendations made here are general and would require site specific adaptations to take into account local and national laws (if applied outside of the UK), the involvement and attitudes of stakeholders, landowners and site managers, and the limitations or constraints of individual conservation projects.

This study has highlighted a weakness of the Mark Release Recapture study system, it is only capable of detecting dispersal occurring at that time and place. In contrast genetic analysis can reveal dispersal that occurred in other years. This is particularly important for a species such as the Marsh Fritillary, where long distance dispersals occur infrequently but still contribute to the genetic connectivity of populations as well as the colonisation of new sites.

This study confirms that Marsh Fritillary disperse more freely than has previously been suggested in the literature, which is based primarily on MRR studies Table 1.1). This is particularly evident in Scotland, where the species disperse across open water distances in excess of the maximum dispersal distance reported in some MRR studies, and in Wales, where there is little differentiation over the western region, showing gene flow is occurring widely and frequently. This should encourage managers to consider populations in the wider landscape context rather than as individual discreet units. It should also prompt those working on similar species where dispersal distances are reported based on MRR studies to consider that dispersal greater than reported may be occurring and may be an important component of the landscape and population dynamics of their species.

6.3 Future work

It is recommended that the further sampling of intermediate habitats described in Chapter 3 be undertaken to determine if the dispersal model suggested, multigenerational stepping-stones, is occurring. Should that be the case, it is likely that the same dispersal pattern occurs in strictly terrestrial habitats which will have further implications for conservation managers, additional work could be done to confirm this.

Further landscape analysis should be carried out to characterise the landscape characteristics which impact gene flow. This will allow managers to better assess where gene flow may be occurring and where best to locate additional habitat or populations to restore gene flow to isolated populations/areas.

Additional investigation of the other populations within the Cumbrian reintroductions would be of interest. This area is unique among Marsh Fritillary populations in that its entire history is known and even if the exact genetic composition

of the founder stock remains unknown, all reintroduced individuals are known to be descended from the same genetic stock. Given the close monitoring that the CMFAG have undertaken the yearly population numbers are also known. This presents an opportunity to identify changes in genetic diversity over time and space, perhaps incorporating potentially functional genes into the study. This will gain additional interest should the parasitoid be reintroduced and natural population dynamics re-establish.

Although beyond the scope of this work, the taxonomy of the Marsh Fritillary in the British Isles should be addressed genetically in the manner of Korb *et al.* (2016) to confirm the presence, absence and status of the various subspecies and forms which have been proposed at one time or another (see Chapter 1 for discussion of the present taxonomic uncertainties). This can be used to inform future conservation translocations within the British Isles and the possible use of the British Isles as a source of *E. aurinia* founders for reintroductions or supplementations to other parts of its range. It is also recommended that an action plan for the species be developed or that the 1995 Species Action Plan (Barnett & Warren,1995) be updated to reflect recent advances in knowledge including those presented in this thesis. This update would establish goals across all four countries of the United Kingdom and ideally also include agreement with the Republic of Ireland for managing the Marsh Fritillary on the Irish landmass.

Beyond the Marsh Fritillary, it is recommended that similar population genetics studies be undertaken on related species, especially where this has not been done previously and where dispersal ability is reported based on MRR methodology. In these cases, long distance dispersals may be occurring too infrequently to be detected in a single year MRR study. Population genetics is able to detect the signature of rare but significant dispersal events and should therefore be applied as broadly as possible, especially to species which are reported to be weak dispersers.

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Appendix A: Molecular approaches.

Each 20µl PCR reaction mix used through thesis contained:

4µl 5x Phusion HF buffer.

0.2µl Phusion DNA polymerase.

0.6µl DMSO.

0.4µl 10mM dNTP mix (containing 2.5nm each of dATP, dCTP, dCTP & dTTP).

1µl 10nM forward primer.

1µl 10nM reverse primer.

2.5µl 10nM $MgCl_2$.

9.3µl Nuclease free water.

1µl Template DNA.

Cycle conditions:

For primers from Smee *et al.* (2013):

1 cycle of 95°C for 5 minutes.

25 cycles of 95°C for 5 seconds, 60°C* for 30 seconds, 68°C for 1 minute.

8 cycles of 95°C for 5 seconds, 53°C* for 30 seconds, 68°C for 1 minute.

1 cycle of 72°C for 30 minutes.

Hold at 4°C

*Temperate increased from 60 to 62°C and 53 to 55°C for Aurnina_16.

For primers from Petenian *et al.* (2005):

1 cycle of 94°C for 5 minutes.

10 cycles of 94°C for 45 seconds, 61°C for 30 seconds, 72°C for 30 seconds.

27 cycles of 94°C for 45 seconds, 51°C* for 30 seconds, 72°C for 45 seconds.

1 cycle of 72°C for 10 minutes.

Hold at 4°C

For primers from (Sinama et al., 2011):

1 cycle of 95°C for 15 minutes.

30 cycles of 94°C for 1 minute, 56°C for 1 minute, 72°C for 1 minute.

1 cycle of 60°C for 45 minutes.

Hold at 4°C

Quality control

All runs included negative controls to check for contamination and positive controls to ensure constancy between runs. 10% of samples were randomly selected and reamplified and re-genotyped to ensure reliability.

Peak scoring.

Automatic peak calling carried out using GeneMapper, this was then checked manually. Clear peaks were defined as 1 or 2 peaks per sample with an amplification at least three times greater than any other peaks (stutter peaks or background noise). Samples with unclear peaks, those with amplification less than three times greater than background or stutter peaks, or where three or more peaks fit the criteria of clear peaks were reamplified and re-genotyped.

For work with preserved specimens in Chapter 5 greater leeway was permitted with defining a clear peak. Clear peaks were defined as 1 or 2 peaks per sample with at least double the amplification of other peaks. Samples which did not have peaks that met these criteria were defined as unclear and treated as above.

Allele binning.

Raw allele scores were binned using TANDEM version 1.07 (Matschiner & Salzburger, 2009) to reduce the potential of human error in the binning process. The program rounds raw allele sizes to integer numbers while taking into account expected nucleotide repeated sizes for that locus. Expected nucleotide repeat sizes were taken from the original papers detailing the development of the specific microsatellite primers.

Appendix B: Tables of allele frequencies by population.

Table B.1. Allele frequencies in Ireland.

Locus	Allele	Site								
		BI	CW	DK	LK	PK	LW	MD	BC	DV
A01	217	0.2037	0.0000	0.1522	0.3889	0.0000	0.2609	0.0556	0.0556	0.0000
	226	0.0556	0.4643	0.0000	0.0185	0.2000	0.0217	0.1944	0.0556	0.1000
	232	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.1000
	235	0.0556	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	238	0.6852	0.5357	0.8478	0.5926	0.8000	0.7174	0.7500	0.8889	0.8000
A13	286	0.0345	0.0000	0.0000	0.0000	0.0000	0.3478	0.1000	0.0000	0.0000
	289	0.8448	0.6964	0.9318	0.7692	0.7069	0.5652	0.7333	0.7750	0.7500
	292	0.1207	0.3036	0.0682	0.2308	0.2931	0.0870	0.1667	0.2250	0.2500
A16	385	0.0000	0.0000	0.1000	0.0200	0.2500	0.0870	0.0000	0.0000	0.0000
	388	0.2778	0.0200	0.0000	0.0400	0.0417	0.0000	0.0278	0.5000	0.0500
	391	0.0185	0.1600	0.4000	0.4200	0.1875	0.2826	0.0833	0.0000	0.1500
	394	0.0185	0.0000	0.0333	0.0000	0.0208	0.0000	0.0556	0.0000	0.0000
	397	0.0370	0.5600	0.1333	0.1400	0.2292	0.1957	0.6389	0.3636	0.6500
	400	0.5926	0.2600	0.3333	0.3800	0.1458	0.1087	0.0278	0.0000	0.0000
	409	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0682	0.0000
	442	0.0000	0.0000	0.0000	0.0000	0.0000	0.0217	0.0000	0.0000	0.0000
	448	0.0556	0.0000	0.0000	0.0000	0.1250	0.3043	0.1667	0.0682	0.1000
	487	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0500

A64	107	0.0000	0.0167	0.0217	0.0000	0.0000	0.0217	0.0000	0.0000	0.0000
	113	0.1429	0.1333	0.1957	0.1429	0.0690	0.2174	0.1111	0.1500	0.6111
	131	0.0000	0.0000	0.0000	0.0179	0.0000	0.0000	0.0000	0.0167	0.0000
	140	0.0476	0.0167	0.0435	0.0179	0.0000	0.1087	0.0000	0.0000	0.0000
	143	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.1833	0.0000
	146	0.5238	0.3333	0.5217	0.5357	0.6897	0.6304	0.5000	0.2667	0.3889
	149	0.0952	0.1167	0.0652	0.0179	0.0000	0.0000	0.0278	0.0000	0.0000
	155	0.1905	0.3833	0.1522	0.2679	0.2414	0.0217	0.3611	0.3833	0.0000

For Site codes see Table 2.1

Table B.2. Allele frequencies in Scotland.

Locus	Allele	Site			
		Islay	Mull	Oronsey	Tayvallich
A01	217	0.0000	0.0000	0.1379	0.2222
	220	0.0000	0.0000	0.0345	0.0000
	223	0.2368	0.0000	0.0172	0.0000
	229	0.1316	0.2308	0.0517	0.0556
	232	0.0000	0.0000	0.1034	0.0000
	235	0.0000	0.0000	0.0000	0.0185
	241	0.0789	0.1538	0.0000	0.0000
	244	0.2105	0.0000	0.0172	0.1111
	247	0.3421	0.5769	0.6379	0.5370
	250	0.0000	0.0385	0.0000	0.0556
A45	185	0.1500	0.0000	0.0185	0.0600
	188	0.4000	0.2917	0.3519	0.5200
	191	0.0500	0.2083	0.2778	0.0800
	194	0.4000	0.3333	0.3333	0.3200
	197	0.0000	0.1667	0.0185	0.0200
A70	110	0.0000	0.0000	0.0000	0.1111
	118	0.0000	0.0000	0.0556	0.3611
	120	0.0000	0.0000	0.0000	0.2500
	124	0.0500	0.5556	0.0556	0.0556
	128	0.9500	0.3889	0.8889	0.2222
	130	0.0000	0.0556	0.0000	0.0000
A16	367	0.0000	0.0000	0.2593	0.0000
	370	0.0000	0.0385	0.0370	0.1786
	385	0.8333	0.8077	0.3889	0.7143
	391	0.0000	0.0769	0.2407	0.0714
	394	0.0278	0.0769	0.0556	0.0000
	400	0.1389	0.0000	0.0185	0.0000
	406	0.0000	0.0000	0.0000	0.0357
Eau88	147	0.0000	0.0000	0.0000	0.0385
	149	0.2857	0.1250	0.5000	0.3846
	151	0.6786	0.7500	0.3000	0.3077
	153	0.0357	0.1250	0.1000	0.0577
	155	0.0000	0.0000	0.1000	0.2115

Table B.3. Allele frequencies in Cornwall.

Locus	Allele	Site			
		Predannack A	Predannack B	Haylekimbro A	Haylekimbro B
A01	229	0.1167	0.0000	0.0862	0.0000
	235	0.0167	0.0000	0.0000	0.0000
	238	0.3667	0.5909	0.2241	0.3333
	241	0.1667	0.0455	0.0172	0.0000
	244	0.3333	0.2500	0.6034	0.5000
	247	0.0000	0.1136	0.0690	0.1667
A13	286	0.1500	0.0455	0.1034	0.0000
	289	0.6000	0.6818	0.6552	0.8333
	292	0.2500	0.2727	0.2414	0.1667
A16	379	0.0000	0.0000	0.0345	0.0000
	391	0.4500	0.4091	0.7069	0.8333
	394	0.0000	0.0455	0.0172	0.0000
	397	0.5500	0.5455	0.2414	0.1667
A64	161	0.9833	1.0000	0.8793	1.0000
	164	0.0167	0.0000	0.1207	0.0000
A70	120	0.0000	0.1765	0.0769	0.0000
	122	0.0400	0.0000	0.0192	0.0000
	126	0.6400	0.7353	0.4423	0.3333
	128	0.0800	0.0294	0.1346	0.0000
	130	0.0600	0.0000	0.0769	0.3333
	132	0.1600	0.0588	0.1923	0.1667
	138	0.0200	0.0000	0.0000	0.0000
	142	0.0000	0.0000	0.0577	0.1667

Table B.4. Allele frequencies in South Wales.

Locus	Allele	Populations										
		A	B	C	D	E	F	G	H	I	J	K
EA26	154	0.6667	0.6389	0.0833	0.4286	1.0000	0.3824	0.5769	0.1875	0.0000	0.2500	0.2500
	156	0.3333	0.1111	0.4167	0.5000	0.0000	0.0882	0.1923	0.0625	0.0000	0.7500	0.1250
	158	0.0000	0.0556	0.1667	0.0000	0.0000	0.1176	0.0769	0.1250	0.5000	0.0000	0.5000
	160	0.0000	0.0000	0.0000	0.0000	0.0000	0.0588	0.0385	0.0625	0.0000	0.0000	0.0000
	162	0.0000	0.0278	0.1667	0.0714	0.0000	0.0294	0.0769	0.1875	0.0000	0.0000	0.0000
	164	0.0000	0.0000	0.0000	0.0000	0.0000	0.0294	0.0000	0.0000	0.0000	0.0000	0.0000
	166	0.0000	0.1667	0.0833	0.0000	0.0000	0.0588	0.0000	0.3750	0.5000	0.0000	0.1250
	168	0.0000	0.0000	0.0000	0.0000	0.0000	0.0588	0.0000	0.0000	0.0000	0.0000	0.0000
	170	0.0000	0.0000	0.0000	0.0000	0.0000	0.0882	0.0385	0.0000	0.0000	0.0000	0.0000
	172	0.0000	0.0000	0.0833	0.0000	0.0000	0.0588	0.0000	0.0000	0.0000	0.0000	0.0000
	180	0.0000	0.0000	0.0000	0.0000	0.0000	0.0294	0.0000	0.0000	0.0000	0.0000	0.0000
EA36	123	0.0000	0.0278	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	125	0.3333	0.2500	0.1667	0.1429	0.0000	0.1176	0.3077	0.2500	0.5000	0.2500	0.1250
	141	0.0000	0.0278	0.0000	0.0714	0.0000	0.0588	0.0000	0.0000	0.0000	0.0000	0.0000
	143	0.6667	0.5278	0.8333	0.6429	1.0000	0.7059	0.6154	0.7500	0.5000	0.5000	0.8750
	145	0.0000	0.0278	0.0000	0.1429	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	147	0.0000	0.1389	0.0000	0.0000	0.0000	0.1176	0.0769	0.0000	0.0000	0.2500	0.0000

EA49	91	0.0000	0.0000	0.0000	0.0000	0.2500	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	93	0.0000	0.0000	0.0000	0.0000	0.0000	0.0588	0.0000	0.0000	0.0000	0.0000	0.0000
	95	0.0000	0.3333	0.0000	0.1429	0.0000	0.0000	0.1538	0.1250	0.0000	0.5000	0.0000
	97	0.5000	0.1944	0.5833	0.3571	0.0000	0.5588	0.3077	0.5000	0.5000	0.0000	0.2500
	99	0.0000	0.0000	0.0000	0.0000	0.0000	0.0588	0.0000	0.0000	0.0000	0.0000	0.0000
	105	0.0000	0.0000	0.0000	0.0714	0.0000	0.0294	0.1923	0.0625	0.0000	0.5000	0.2500
	133	0.0000	0.1944	0.0000	0.1429	0.0000	0.0588	0.0000	0.1250	0.0000	0.0000	0.0000
	135	0.0000	0.0278	0.2500	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.5000
	137	0.1667	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	149	0.0000	0.0000	0.0000	0.0000	0.5000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	153	0.0000	0.0000	0.0000	0.1429	0.0000	0.0294	0.1538	0.0000	0.0000	0.0000	0.0000
	155	0.0000	0.0000	0.0000	0.1429	0.0000	0.0588	0.0000	0.1250	0.0000	0.0000	0.0000
	157	0.3333	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0625	0.0000	0.0000	0.0000
	159	0.0000	0.2500	0.1667	0.0000	0.0000	0.1176	0.1538	0.0000	0.1667	0.0000	0.0000
	161	0.0000	0.0000	0.0000	0.0000	0.0000	0.0294	0.0385	0.0000	0.3333	0.0000	0.0000
	163	0.0000	0.0000	0.0000	0.0000	0.2500	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000

Eau21	101	0.0000	0.0000	0.0000	0.1429	0.0000	0.0000	0.0769	0.0625	0.0000	0.5000	0.0000
	105	0.0000	0.1111	0.0000	0.1429	0.5000	0.0588	0.2308	0.3125	0.3333	0.0000	0.0000
	109	0.0000	0.0000	0.0000	0.0000	0.5000	0.1176	0.0769	0.1250	0.0000	0.2500	0.2500
	119	0.0000	0.0000	0.0000	0.1429	0.0000	0.0000	0.0385	0.0000	0.0000	0.0000	0.0000
	121	0.1667	0.1667	0.0000	0.0000	0.0000	0.2647	0.1154	0.1875	0.3333	0.2500	0.0000
	123	0.5000	0.4167	0.4167	0.1429	0.0000	0.0882	0.1923	0.2500	0.0000	0.0000	0.3750
	125	0.0000	0.1111	0.3333	0.2857	0.0000	0.2059	0.0769	0.0625	0.3333	0.0000	0.0000
	127	0.0000	0.1111	0.0833	0.0000	0.0000	0.1765	0.0769	0.0000	0.0000	0.0000	0.1250
	129	0.3333	0.0833	0.1667	0.0714	0.0000	0.0294	0.0385	0.0000	0.0000	0.0000	0.1250
	131	0.0000	0.0000	0.0000	0.0714	0.0000	0.0588	0.0769	0.0000	0.0000	0.0000	0.1250
Eau32	269	0.3333	0.0000	0.0833	0.2143	0.2500	0.0000	0.1923	0.1250	0.0000	0.0000	0.7500
	271	0.5000	0.6389	0.8333	0.4286	0.5000	0.7647	0.4231	0.1875	0.8333	0.2500	0.1250
	275	0.1667	0.2222	0.0000	0.1429	0.2500	0.2059	0.3462	0.3125	0.1667	0.2500	0.0000
	277	0.0000	0.1389	0.0833	0.2143	0.0000	0.0294	0.0385	0.3750	0.0000	0.5000	0.1250
Eau45	142	0.5000	0.1389	0.1667	0.0000	0.0000	0.0000	0.0385	0.0000	0.0000	0.0000	0.0000
	152	0.3333	0.6944	0.5833	0.4286	0.5000	0.6765	0.7308	0.6875	0.5000	0.2500	0.3750
	154	0.1667	0.0278	0.0833	0.0714	0.5000	0.0588	0.0385	0.0625	0.0000	0.2500	0.0000
	156	0.0000	0.0278	0.0833	0.2857	0.0000	0.0882	0.0769	0.1875	0.0000	0.5000	0.0000
	158	0.0000	0.0278	0.0000	0.0000	0.0000	0.0882	0.0385	0.0625	0.1667	0.0000	0.2500
	160	0.0000	0.0833	0.0833	0.2143	0.0000	0.0882	0.0769	0.0000	0.3333	0.0000	0.3750

Eau64	146	0.0000	0.0833	0.2500	0.5714	0.0000	0.2353	0.2308	0.1875	0.0000	0.5000	0.0000
	148	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0385	0.0000	0.0000	0.0000	0.0000
	150	0.0000	0.0278	0.0000	0.0000	0.0000	0.0000	0.0385	0.0000	0.3333	0.0000	0.0000
	152	0.3333	0.4444	0.0833	0.0714	0.0000	0.2941	0.2308	0.1875	0.0000	0.0000	0.6250
	154	0.0000	0.3889	0.3333	0.3571	0.5000	0.4118	0.4615	0.6250	0.6667	0.5000	0.3750
	158	0.3333	0.0000	0.0833	0.0000	0.5000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	160	0.0000	0.0556	0.2500	0.0000	0.0000	0.0588	0.0000	0.0000	0.0000	0.0000	0.0000
	162	0.3333	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Eau71	117	0.3333	0.6667	0.2500	0.5714	1.0000	0.6765	0.5769	0.6875	0.3333	1.0000	0.6250
	119	0.6667	0.3333	0.6667	0.4286	0.0000	0.3235	0.4231	0.3125	0.6667	0.0000	0.3750
	125	0.0000	0.0000	0.0833	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Eau72	133	0.0000	0.0278	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	135	0.0000	0.4722	0.5000	0.5714	0.5000	0.8235	0.6154	0.6250	1.0000	0.0000	0.5000
	161	1.0000	0.1944	0.2500	0.1429	0.2500	0.0882	0.0385	0.0000	0.0000	0.0000	0.0000
	163	0.0000	0.1667	0.0000	0.0714	0.0000	0.0000	0.0000	0.0000	0.0000	0.2500	0.0000
	165	0.0000	0.0833	0.2500	0.2143	0.2500	0.0882	0.3462	0.1875	0.0000	0.5000	0.5000
	167	0.0000	0.0556	0.0000	0.0000	0.0000	0.0000	0.0000	0.1875	0.0000	0.2500	0.0000

Eau73	151	0.0000	0.0278	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	153	0.0000	0.0000	0.0000	0.0000	0.0000	0.0294	0.0000	0.0000	0.0000	0.0000	0.0000
	155	0.0000	0.5556	0.8333	0.8571	1.0000	0.6176	1.0000	0.6250	1.0000	0.0000	0.8750
	157	0.0000	0.0000	0.0000	0.0000	0.0000	0.0588	0.0000	0.0625	0.0000	0.0000	0.1250
	161	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0625	0.0000	0.0000	0.0000
	163	1.0000	0.4167	0.1667	0.1429	0.0000	0.2059	0.0000	0.2500	0.0000	1.0000	0.0000
	165	0.0000	0.0000	0.0000	0.0000	0.0000	0.0882	0.0000	0.0000	0.0000	0.0000	0.0000
Eau81	152	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0769	0.0625	0.0000	0.0000	0.0000
	154	0.0000	0.0000	0.0000	0.1429	0.0000	0.0000	0.0000	0.1250	0.0000	0.0000	0.0000
	158	0.0000	0.0556	0.0000	0.0000	0.0000	0.0294	0.0000	0.0000	0.0000	0.0000	0.0000
	162	0.3333	0.3889	0.7500	0.7143	0.0000	0.6471	0.6154	0.2500	0.3333	0.5000	0.3750
	164	0.0000	0.0000	0.0000	0.0000	0.0000	0.0294	0.0000	0.0625	0.0000	0.0000	0.0000
	170	0.6667	0.5556	0.2500	0.1429	1.0000	0.2941	0.3077	0.5000	0.6667	0.5000	0.6250
Eau88	153	0.0000	0.0833	0.0000	0.0000	0.0000	0.0000	0.0000	0.3750	0.0000	0.0000	0.0000
	155	1.0000	0.5833	1.0000	0.2857	0.7500	0.6471	0.5385	0.4375	0.6667	0.0000	0.7500
	157	0.0000	0.3333	0.0000	0.7143	0.0000	0.3529	0.3462	0.0625	0.3333	1.0000	0.2500
	159	0.0000	0.0000	0.0000	0.0000	0.2500	0.0000	0.1154	0.1250	0.0000	0.0000	0.0000

For Population codes see Figure 4.

Table B.5. Allele frequencies in Cumbria.

Locus	Allele	Population					
		B	E	F	M	1980s	1920s
A01	220	0.0000	0.0000	0.0179	0.0000	0.0000	0.0000
	240	0.0667	0.0000	0.1250	0.1957	0.0000	0.0000
	243	0.8333	0.9655	0.8571	0.8043	0.0000	0.0000
	249	0.1000	0.0345	0.0000	0.0000	0.0000	0.0000
	275	0.0000	0.0000	0.0000	0.0000	0.0000	0.1250
	278	0.0000	0.0000	0.0000	0.0000	0.5500	0.3750
	281	0.0000	0.0000	0.0000	0.0000	0.1750	0.1875
	287	0.0000	0.0000	0.0000	0.0000	0.2500	0.1250
	290	0.0000	0.0000	0.0000	0.0000	0.0250	0.1875
A45	182	0.0000	0.0000	0.0000	0.0000	0.3636	0.0833
	185	0.1333	0.1786	0.2115	0.0870	0.0000	0.1667
	188	0.0000	0.0536	0.0000	0.0000	0.0455	0.1667
	194	0.1167	0.0000	0.0192	0.2826	0.0909	0.0833
	197	0.1667	0.1607	0.1346	0.0870	0.2727	0.3333
	200	0.3167	0.2143	0.1346	0.4348	0.0909	0.0000
	203	0.0500	0.1250	0.0769	0.0652	0.0909	0.1667
	206	0.1333	0.0000	0.1346	0.0435	0.0455	0.0000
	212	0.0833	0.1250	0.2885	0.0000	0.0000	0.0000
	218	0.0000	0.0179	0.0000	0.0000	0.0000	0.0000
	221	0.0000	0.1250	0.0000	0.0000	0.0000	0.0000
A70	118	0.3448	0.2586	0.3929	0.2955	0.5000	0.4286
	122	0.3276	0.1897	0.5000	0.4773	0.5000	0.3571
	124	0.0172	0.0000	0.0179	0.0000	0.0000	0.0000
	128	0.2931	0.5517	0.0893	0.2273	0.0000	0.1429
	130	0.0172	0.0000	0.0000	0.0000	0.0000	0.0000
	138	0.0000	0.0000	0.0000	0.0000	0.0000	0.0714
Eau88	133	0.0833	0.0345	0.1833	0.0652	0.3000	0.4375
	135	0.0833	0.2241	0.0667	0.0000	0.0250	0.0000
	141	0.0000	0.0345	0.1167	0.0217	0.0500	0.0000
	145	0.0333	0.0000	0.0000	0.0870	0.0500	0.0000
	147	0.1167	0.0345	0.0500	0.0870	0.1250	0.0625
	151	0.1500	0.2586	0.4000	0.0000	0.0250	0.0625
	153	0.0333	0.1207	0.0167	0.0870	0.0000	0.0000
	157	0.4667	0.1897	0.0500	0.5000	0.3750	0.4375
	159	0.0333	0.1034	0.1167	0.1522	0.0500	0.0000

A13	205	0.1207	0.0172	0.0167	0.3636	NA	NA
	208	0.0345	0.0172	0.0000	0.0455	NA	NA
	229	0.0345	0.0000	0.0000	0.0000	NA	NA
	232	0.1034	0.0000	0.0333	0.0455	NA	NA
	271	0.0000	0.0000	0.0000	0.0455	NA	NA
	280	0.0517	0.0345	0.0000	0.0455	NA	NA
	283	0.5862	0.7586	0.7833	0.4091	NA	NA
	286	0.0517	0.1724	0.1333	0.0455	NA	NA
	292	0.0000	0.0000	0.0333	0.0000	NA	NA
	316	0.0172	0.0000	0.0000	0.0000	NA	NA

Population codes: B, Braithwaite. E, Ennerdale. F, Finglandrigg. M, Middlesceugh. 1980s, specimens collected 1977-1993. 1920s, specimens collected 1920-1930.

Appendix C – Detailed regional climate data

Data is from Met Office climate monitoring stations and represents the average values for 1981-2010 unless otherwise stated. Temperatures and windspeed are means for the period in question. Rainfall, frost and hours of sunshine are totals for the period in question.

C.1 Scotland

Data from Port Ellen climate station (Location: 55.681, -6.256. Elevation 17m) (Met Office, n.d.-c).

Month	Maximum temperature (°C)	Minimum temperature (°C)	Days of air frost (days)	Rainfall (mm)	Days of rainfall ≥1 mm (days)	Mean wind speed at 10 m (knots)
January	7.8	2.6	6.7	138.2	19.9	15.2
February	7.6	2.2	6.7	98.2	14.8	14.4
March	9.1	3.1	4.8	118.8	17.8	14
April	11	4.2	2.6	77.9	13.3	12.2
May	13.9	6.4	0.5	62.4	11.6	11.8
June	15.8	8.9	0	73.3	11.4	10.5
July	17.1	10.9	0	78.8	13.8	10.1
August	17.1	11	0	106.7	15.4	10.4
September	15.6	9.6	0	114.6	15.5	11.7
October	12.9	7.5	0.9	148.7	19.7	13.2
November	10	4.9	3.1	132.3	19.3	13.7
December	8.2	3	6.5	132.9	18.6	13.4
Annual	12.2	6.2	31.9	1282.7	191.1	12.5

C.2 Cornwall

Data from Culdrose climate station (Location: 50.085, -5.257. Elevation 78m) (Met Office, n.d.-b).

Month	Maximum temperature (°C)	Minimum temperature (°C)	Days of air frost (days)	Maximum sunshine (hours)	Rainfall (mm)	Days of rainfall ≥1 mm (days)	Mean wind speed at 10 m (knots)
January	9.2	4.1	4.4	56.6	113.4	16.5	14
February	9	3.7	4.2	79.7	80.4	12.4	13.1
March	10.2	4.8	1.8	109.7	80.2	13	12.8
April	11.9	5.6	0.7	177.8	67.2	11.6	11.5
May	14.6	8.3	0	205.9	60	9.9	11.5
June	17.1	10.7	0	202.7	60.5	8.7	10.1
July	19	12.8	0	197.2	61.8	10	10
August	19.2	12.9	0	192	67	10.7	9.6
September	17.4	11.2	0	150.4	71.9	10.2	10.5
October	14.4	9.2	0.1	105.7	105	15.2	12.2
November	11.7	6.6	0.7	76.3	115.7	15.8	12.6
December	9.9	4.8	2.9	54.2	115.9	16.2	13.8
Annual	13.7	7.9	14.8	1607.9	998.8	150.3	11.8

C.3 South Wales

Data from Mumbles Head climate station (Location: 51.565, -3.981. Elevation 32m) (Met Office, n.d.-e).

Month	Maximum temperature (°C)	Minimum temperature (°C)	Days of air frost (days)	Rainfall (mm)	Days of rainfall ≥1 mm (days)	Mean wind speed at 10 m (knots)
January	8	4	3	95.5	15	15.5
February	7.8	3.6	3.3	67	11	14.3
March	9.5	4.8	0.7	72.9	13.5	13.9
April	11.9	6.3	0.1	58.5	10.6	12.2
May	15	9.2	0	62.8	10.3	12.4
June	17.7	11.8	0	63.8	9.9	10.8
July	19.6	13.9	0	71.9	10.1	11.7
August	19.7	14	0	83.9	11.2	11.8
September	17.8	12.4	0	77.4	11.4	12.9
October	14.4	9.9	0	123.1	15.4	15.1
November	11.1	6.9	0.3	112.1	15	14.3
December	8.7	4.7	2.3	110.3	14.4	15.4
Annual	13.5	8.5	9.7	999.2	147.9	13.3

C.4 Cumbria

Data from Keswick climate station (Location: 54.614, -3.157. Elevation 81M) (Met Office, n.d.-d).

Month	Maximum temperature (°C)	Minimum temperature (°C)	Days of air frost (days)	Rainfall (mm)	Days of rainfall ≥1 mm (days)	Mean wind speed at 10 m (knots)
January	7.2	1.6	11	169.1	16.8	7.1
February	7.4	1.4	11.2	119.9	13.1	7
March	9.4	2.8	7	127.8	15.8	7
April	11.9	4.2	4.8	81.7	12.5	5.7
May	15.6	6.4	1.3	79.4	12	5
June	17.9	9.3	0.1	84.3	12.2	4
July	19.7	11.5	0	88.1	12.9	3.5
August	19.1	11.1	0	104.1	14.4	3.5
September	16.7	9	0.2	126.6	13.9	4.1
October	13.3	6.7	2.4	189.3	17.8	4.8
November	9.7	3.9	6.4	177.9	17.7	5.9
December	7.5	1.5	12	173	17	6.2
Annual	13	5.8	56.5	1521	176.1	5.3

C.5 Ireland

Climate data for Irish sites by county (corresponding sites codes in brackets), data from the Met Éireann (n.d.) except for County Down which is from the Met Office (n.d.-a).

County Cork (BC)				
Station Details	Name: Roches. Location: 51.793/-8.244. Elevation:43m. Average years: 1971-2000			
Month	Maximum temperature (°C)	Minimum temperature (°C)	Rainfall (mm)	Days of rainfall ≥1 mm (days)
January	8.8	4.2	79.5	11
February	8.8	4.4	72	9
March	9.9	4.9	63.9	9
April	11.7	5.8	39.7	6
May	13.9	8.1	50.6	8
June	16.6	10.5	43.3	7
July	18.6	12.4	42.4	6
August	18.5	12.5	61.8	7
September	16.5	11	57.2	7
October	13.8	9.1	79.8	9
November	11	6.4	60.6	9
December	9.6	5.2	77.3	10
Annual	13.2	7.9	727.9	98

County Westmeath (CW, LD)				
Station Details	Name: Mullingar. Location: 53.537/-7.362. Elevation: 101m. Average years: 1979-2008			
Month	Maximum temperature (°C)	Minimum temperature (°C)	Rainfall (mm)	Days of rainfall ≥1 mm (days)
January	7.4	1.5	91.7	15
February	7.9	1.5	72	13
March	9.8	2.8	78.3	15
April	12.1	4.1	62.1	11
May	14.9	6.3	68.7	12
June	17.3	9.2	70.5	11
July	19.2	11.1	61.8	11
August	18.9	10.8	80.8	13
September	16.7	8.9	73.8	12
October	13.2	6.2	102.1	14
November	9.9	3.5	82.4	13
December	7.9	2.2	97.1	14
Annual	12.9	5.7	941.3	154

County Kildare (DK, LK, PK, LW)				
Station Details	Name: Casement. Location: 53.301/-6.451. Elevation 97m. Average years: 1981-2010			
Month	Maximum temperature (°C)	Minimum temperature (°C)	Rainfall (mm)	Days of rainfall ≥1 mm (days)
January	8	2.1	63.8	12
February	8.2	2	48.5	10
March	10.2	3.3	50.7	11
April	12.4	4.1	51.9	10
May	15.2	6.6	59.1	11
June	17.9	9.4	62.5	10
July	19.8	11.5	54.2	10
August	19.5	11.3	72.3	11
September	17.1	9.5	60.3	10
October	13.6	7	81.6	12
November	10.2	4.2	73.7	11
December	8.3	2.4	75.7	12
Annual	13.4	6.1	754.2	130

County Tipperary (DV)				
Station Details	Name: Shannon Airport. Location: 52.702/-8.924. Elevation: 1m. Average years: 1981-2010			
Month	Maximum temperature (°C)	Minimum temperature (°C)	Rainfall (mm)	Days of rainfall ≥1 mm (days)
January	8.8	3.2	102.3	16
February	9.2	3.2	76.2	12
March	11.1	4.5	78.7	14
April	13.3	5.7	59.2	11
May	16	8.2	64.8	12
June	18.3	10.9	69.8	11
July	19.8	12.9	65.9	12
August	19.6	12.7	82	13
September	17.7	10.8	75.6	12
October	14.3	8.2	104.9	16
November	11.1	5.5	94.1	15
December	9	3.6	104	15
Annual	14	7.4	977.6	159

Dublin (BI)				
Station Details	Name: Dublin Airport. Location: 53.430/-6.250. Elevation: 22m. Average years: 1981-2010			
Month	Maximum temperature (°C)	Minimum temperature (°C)	Rainfall (mm)	Days of rainfall ≥1 mm (days)
January	8.1	2.4	62.6	12
February	8.3	2.3	48.8	10
March	10.2	3.4	52.7	11
April	12.1	4.6	54.1	10
May	14.8	6.9	59.5	11
June	17.6	9.6	66.7	10
July	19.5	11.7	56.2	10
August	19.2	11.5	73.3	11
September	17	9.8	59.5	10
October	13.6	7.3	79	11
November	10.3	4.5	72.9	11
December	8.3	2.8	72.7	12
Annual	13.3	6.4	758	129

County Down (MD)				
Station Details	Name: Castlederg. Location: 54.707/-7.577. Elevation: 49m. Average years: 1981-2010			
Month	Maximum temperature (°C)	Minimum temperature (°C)	Rainfall (mm)	Days of rainfall ≥1 mm (days)
January	7.4	1.3	127.8	18.7
February	7.9	1.2	93.3	15.3
March	9.9	2.4	98.1	17.2
April	12.2	3.6	74.6	13.7
May	15.3	5.7	65.8	13.6
June	17.4	8.9	66	12.6
July	18.9	10.8	83.5	14
August	18.6	10.5	85.1	15.1
September	16.6	8.7	91.5	14.6
October	13.2	5.9	122.6	17.7
November	9.8	3.3	110.9	17.8
December	7.4	1.2	124.6	17.6
Annual	12.9	5.3	1143.7	187.7

Appendix D

List of the microsatellites and their respective sources used by Nevada Genomics for the Marsh Fritillary samples from south Wales.

From Petenian *et al.* (2005):

- EA26
- EA36
- EA49

From Sinama *et al.* (2011):

- Eau21
- Eau32
- Eau45
- Eau52
- Eau59
- Eau64
- Eau71
- Eau72
- Eau73
- Eau81
- Eau88