Genetic population structure of the Scotch argus butterfly (*Erebia aethiops*) in Britain: implications for conservation and future reintroductions

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LEC

Dedicated to my late father, Tony Gunson who told me to follow my own path and that my best would always be good enough. Thank you for always being on my side Dad.

Declaration

This thesis has not been submitted in support of an application for another degree at this or any other university. It is the result of my own work and includes nothing that is the outcome of work done in collaboration except where specifically indicated. Many of the ideas in this thesis were the product of discussion with my supervisors Dr Rosa Menendez Martinez and Dr Mike Roberts.

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Abstract

Along with many other species, the Scotch argus (*Erebia aethiops*) has suffered a decline in range, particularly in England, with some populations experiencing long-term isolation. This thesis studied the genetic structure of the species in Britain on a national scale and at the metapopulation level with the aim of advising future management of the species and potential reintroductions across its former range in England.

AFLP analysis carried out on populations from England, Scotland and the western Scottish islands found that there was no significant difference in genetic diversity between the regions. None of the study populations showed clear signs of inbreeding, suggesting inbreeding depression (a reduction in fitness due to inbreeding) is not a concern. Even those populations in England which were probably isolated for a long time (e.g. Arnside Knott) showed genetic diversity levels that were relatively high. However, populations were genetically differentiated with significant differences observed among both regions and populations. Genetic differences among populations were significantly related to geographic distance.

On a local scale, the Smardale Gill metapopulation was found to be genetically robust with gene flow occurring between all patches. This was confirmed with a mark-release-recapture study which show that males are able to move long distances and the total population estimate for the whole area was high (over 7000 individuals). However, females moved only small distances, and none were reported to move between patches, suggesting that gene flow between patches is only maintained by males and colonisation of empty patches is limited.

The results of this study provide support for several management recommendations for the conservation of the species. The struggling Arnside Knott population at the most southern range margin was found not to be suffering from inbreeding, so it is recommended that no supplementary translocations be made until the cause of the decline is determined. As populations were genetically differentiated, a geographically close population is recommended as a source to increase the chance of success of any future reintroduction. In this respect the Smardale Gill metapopulation appears the most suitable source for future reintroduction attempts across the species' former range in England.

1. Introduction

1.1 Biodiversity loss and extinctions

Many animal and plant species have experienced a dramatic decline in recent decades (Pimm et al. 1995; Baillie et al. 2004; Stuart et al. 2004), leading some scientists to suggest we are entering a sixth mass extinction event (Ceballos et al. 2010; Barnosky et al. 2011), the largest in 65 million years (Lawton & May 1995). Current extinction rates are estimated to be around 1000 times higher than the background rate observed in fossil records (Cushman 2006).

Climate change is a major factor in biodiversity loss (Thomas et al. 2004), with anthropogenic global warming increasing the rate of change more rapidly than can be compensated for by evolutionary adaptation (Mayhew et al. 2008). The last century has seen a global temperature increase of 0.85°C (IPCC 2014). As climatic isotherms move upwards in altitude and towards the poles , there is evidence that many taxa will follow suit (Parmesan 2006; Thomas et al. 2006), including Lepidoptera (Parmesan et al. 1999).

Habitat destruction and fragmentation due to changes in land use are also major contributors to biodiversity loss and range reduction (Brooks et al. 2002; Dobson et al. 2006). As well as maintaining connectivity across a fragmented landscape (Hanski & Gaggiotti 2004), ensuring the quality of the remaining habitat patches is also essential to species' survival (Thomas et al. 2004, Ellis et al. 2012). The combination of both climate change and habitat loss creates a greater impact than either individual threat (Mantyka-Pringle et al. 2012).

Vertebrates receive the most attention in the literature covering extinctions and declines (Wake & Vredenburg 2008; l 2015; Ceballos et al. 2017), while invertebrates are sometimes overlooked. Dunn (2005) estimates that <1% of all insect extinctions have been documented. Additionally, it has been suggested that some insect species may go extinct without notice, due to the lack of monitoring and identification prior to extinction (Eisenhauer et al. 2019)

Butterflies are slightly better represented in the scientific literature than other insects and their decline has been the subject of recent study (Fox et al. 2007; van Swaay et al. 2008; 2015; Warren 1997). This may be a result of their popularity as an indicator species (Sawchik 2005; van Swaay & van Strien 2008), partly due to their sensitivity to environmental changes (Kremen 1992; Warren et al. 2001).

The use of insects as model species is common (Roy & Wajnberg 2008). Butterfly population assessment has been employed as a method of determining habitat quality (Kremen 1992), a predictor of species richness (Fleishman et al. 2005) and as an indicator of the impacts of climate change (Vickery 2008). Butterfly richness has also

been found to correlate with the abundance of threatened or endangered species from higher taxa (Bonebrake et al. 2010).

The literature shows that some species of Lepidoptera are still declining in the UK (Thomas et al. 2004; Ellis et al. 2012; Fox et al. 2015) despite a recent increase in conservation efforts (Fox et al. 2015). Habitat specialists have suffered the most (Krauss et al. 2003) but generalists have also experienced an overall reduction in numbers and range (Fox et al. 2007).

1.2 Fragmentation and isolation

Habitat destruction and degradation can lead to isolated patches of suitable habitat within an otherwise heterogenous landscape. Habitat loss and fragmentation affects species on all trophic levels (Rahel et al. 1996; Young et al. 1996; Tian et al. 2014), and most strongly impacts species with low dispersal capabilities (Cushman 2006). Although populations may naturally become fragmented or isolated due to environmental pressures (Stevens & Hogg 2003) or adaptation (Svensson et al. 2006), isolation via habitat fragmentation/destruction can also be caused by anthropogenic influences such as changes in land use (Fahrig 2003).

Some species can persist in a fragmented landscape by forming a metapopulation; a set of smaller populations of the same species which are geographically separate but linked via dispersal (Hanski & Gaggiotti 2004). If the patches are sufficiently connected, a declining patch may experience a 'rescue effect', whereby individuals from a thriving patch disperse to supplement or recolonise it, stabilising the metapopulation as a whole (Gonzalez et al 1998). In this way, a metapopulation can also increase its size (Hanski 1999) and genetic diversity (Saccheri et al. 1998).

The effects of fragmentation are not always immediately apparent. Populations may survive for long periods before going extinct (Brook et al. 2008; Krauss et al. 2010), creating an extinction debt, whereby a species survives fragmentation but goes extinct later without additional changes. This can lead to the underestimation of the threat facing a species. (Kuussaari et al. 2009). This effect can also allow a 'grace' period during which connectivity can be restored before extinction occurs, providing the threat is recognised in time (Krauss et al. 2010).

Functional connectivity may be facilitated by artificially created corridors or stepping stones within the fragmented environment, providing they are tailored to the target species, to allow permanent dispersal (Bennett 1990) or temporary mating excursions (Aars 1999). For species which can traverse a corridor in a single generation, such as butterflies, the corridor may be of significantly lower quality than the patch habitat while still increasing gene flow and dispersal throughout the metapopulation (Lehtinen et al. 1999; Haddad & Tewksbury 2005).

As well as understanding a target species' habitat requirements, successful conservation and management also requires an understanding of behaviour. If a species can travel freely through fragmented landscapes, no corridors or stepping stones are necessary as the patches are already functionally connected (With 1997). Additionally, differences between male and female behaviour may influence rates of dispersal and corridor use (Pusey 1987), such as male-biased dispersal for patrolling or mating (Trochet at al. 2013) and female-biased dispersal due to male harassment at high densities (Baguette et al 1998). Such differences should be considered when assessing the functional connectivity for the species.

1.3 Genetic diversity and differentiation

Long term isolation or restricted connectivity can reduce or eliminate gene exchange and prevent the addition of new genetic material to the population, increasing the risk of inbreeding depression (Andersen et al. 2004); a major cause of fitness reduction which is especially damaging to small populations (Frankham 1995). Inbreeding depression occurs when closely related individuals mate to produce offspring and is dependant on natural selection, genetic drift and past mutations (Hedrick & Garcia-Dorado 2016.). However, inbreeding does not always lead to inbreeding depression.

While some species naturally regulate their mating behaviour to avoid inbreeding - (Stow & Sunnucks 2004), inbreeding has been shown to reduce fitness in Lepidoptera (Saccheri 1996) and negatively impacts survival and longevity at all life stages (Saccheri 1998). Determining whether inbreeding levels are relative to population size allows conclusions to be made regarding mating behaviour of the species.

Restricted connectivity and gene flow also makes the population more susceptible to deleterious alleles persisting in the homozygous form (Zachos et al. 2007). However, the opposite can also be seen, whereby isolated population are protected from purged deleterious alleles which would otherwise have been reintroduced via immigration (Keller & Waller 2002), highlighting the importance of connectivity restoration only after assessment of all potentially connecting populations.

The Founder Effect can result from isolation if only a small number of individuals are present in the population at the time of separation (Provine 2004). As well as promoting inbreeding, this effect has been suggested to cause rapid speciation, particularly in short-lived species such as insects (Templeton 1980), although some insect species have proven resistant to founder-led speciation (Moya et al. 1995).

Speciation due to isolation can also occur when the environmental conditions differ between the isolated areas and the groups adapt to exploit different niches (Rice 1987). Differences in behaviour or physiology may mean that populations are no longer able to breed, even if connectivity is re-established. It also means that translocated individuals must be taken from a sufficiently similar population if they are to be used to supplement the gene pool of another.

Potentially, isolation could lead to beneficial adaptations. A small population may adapt more quickly to pressures such as climate change (Keller & Seehausen 2012) and become fitter as a result; able to survive in a changing environment and possibly serve as a source for future reintroduction. Genetic differentiation is a possible indicator of such adaptations and can also serve as an indicator of progressive speciation (Ayala et al. 1974).

1.4 Reintroduction and translocation for conservation

The goal of reintroduction or assisted translocation is the establishment of a self-sustaining population of the extirpated species within its previous range, or movement of individuals to supplement an existing population. This may be as part of a conservation effort (Griffith et al. 1989), ecological management (Linnell et al. 1997) or to supplement hunting stock (Fischer & Lindenmayer 2000).

Reintroduction biology is a relatively new field with one of the earliest documented reintroductions being that of the American bison in 1907 (Kleiman 1989). The awareness of reintroduction as a viable conservation tool has increased and success rates are improving. In the 1970s and 1980s the majority of documented reintroduction attempts did not succeed past the first several years (Griffith et al. 1989; Wolf et al. 1996; Seddon et al. 2007) and this prompted the IUCN's creation of the Reintroduction Specialist Group and a set of guidelines for reintroductions and other conservation translocation (IUCN/SSC 1998). The updated guidelines (IUCN 2013) emphasise the need for feasibility assessments and extensive background knowledge before any form of translocation is attempted, followed by comprehensive monitoring and documentation.

Reintroduction attempts typically favour endangered species (Allen 1994; Short et al. 1994; Pearce & Lindenmayer 1998) or those which provide an ecological service (Miller et al. 1999; Hedrick & Fredrickson 2008) with founding or additional individuals taken from the wild (Armstrong et al. 199) or bred in captivity (Bremner-Harrison et al. 2004).

Mammal reintroductions such as the black-footed ferret (Miller et al. 1994) and the Yellowstone wolves (Fritts et al. 1997) are well documented and capture the public interest (Bath 1989). However, insect reintroductions are becoming more common as their importance to the overall health of an ecosystem is better understood (Greenwood 1987; Corbet et al. 1991; Losey & Vaughan 2006).

One of the most well-documented UK insect reintroductions is the large blue (*Maculinea arion*) butterfly, which went extinct in the UK in 1979 and was reintroduced using a Swedish source population. Andersen et al. (2014) found that there was no reduction in genetic diversity among the reintroduced populations in 2011, however there was already evidence of genetic differentiation indicated by the presence of several private alleles not found in the Swedish populations.

Another UK butterfly reintroduction success story is the Marsh fritillary (*Euphydryas aurinia*) project, which mixed native Cumbrian individuals with those taken from Scottish populations for captive breeding. This was done partly due to the extremely low English numbers; only 95 caterpillars were recovered from the last remaining Cumbrian colony (Porter & Ellis 2011). The Scottish individuals were also added to boost the genetic diversity of the English *E. aurinia* population and were able to mate successfully with the English individuals (Smee 2011). Mixing source populations has the potential to lead to outbreeding depression in the hybrid offspring (Huff et al. 2011) but has generally worked well in butterfly reintroductions.

Reintroduction and translocation attempts are not always successful and the reasons can vary. Insufficient habitat restoration and/or management can lead to dispersal and survival failure (Bennett et al. 2013) as can predation from invasive (Moseby et al. 2011) or feral (Hardman et al. 2016) species already present at the release site or native predators in sufficient numbers (Grey-Ross et al. 2009). Overall, the evidence suggests that reintroduction attempts are becoming more successful but failure is still a more likely outcome, partly dependant on robust planning and surveying prior to the release.

1.5 Scotch argus (*Erebia aethiops*) status

The *Erebia* genus is comprised of >90 species (Tennent 2008), most of which inhabit boreal or alpine environments (Slamova et al. 2010). However, along with *E. medusa*, *E, aethiops* can be found in warmer lowland regions of grassland or sparse woodland which tend to be more heterogenous than the mountainous range of congeners (Asher et al. 2001).

Part of the *Nymphalidae* butterfly family, *E. aethiops* is a northerly distributed species with a retracting southern range (Franco et al. 2006). It is found on limestone grassland and young woodland and is easily identified by dark wings with a distinctive orange band and brown spots. Males and females look similar but are distinguishable by lighter, dusty brown wings in the female along with a fatter body (Figure 1.1). They also display slight behavioural differences with the males emerging earlier and flying higher (Kirkland 2012).

Figure 1.1. Mating pair of *Erebia aethiops* taken at Smardale Gill Nature Reserve. Male is on the right, female on the left (photo source: Tom Dunbar).

Despite the name, the Scotch argus is also found in England along with parts of Europe and Asia. *E. aethiops*, along with most other UK butterfly species, has experienced a range contraction in the last century (Fox et al. 2007), and the English distribution is limited to two major populations (Asher et al. 2001). There are also two smaller English populations, Crosby Garrett and Bastow Wood but it is uncertain whether these are genuine populations which have survived the retraction or whether they are unauthorised reintroductions.

E. aethiops' range retraction may be a result of habitat fragmentation and degradation (Slamova et al. 2013) and/or climate change (Hill et al. 2001; Franco et al. 2006; Menéndez et al. 2007) or a combination of both. The species, which was once common in Cumbria, Yorkshire, Lancashire, and Durham (Lucas 1893; South 1928) is now only found in significant numbers at two English sites (Fox et al. 2015), Arnside Knott and Smardale Gill in Cumbria, although it is still abundant throughout Scotland (Figure 1.2).

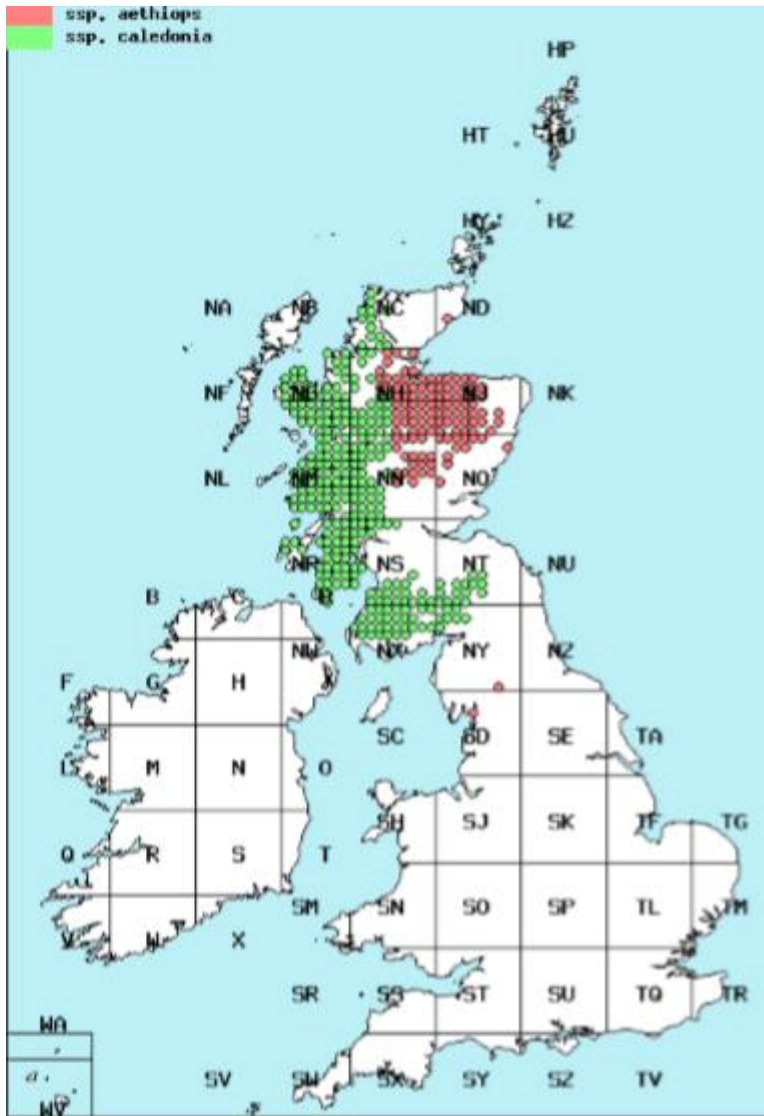

Figure 1.2. Distribution and subspecies separation of *Erebia aethiops* in the UK. The *aethiops* subspecies is in red and the *caledonia* is in green (Butterfly-conservation.org 16/11/18).

Although overall UK abundance of *E. aethiops* has increased in the last few years, occurrences have decreased (Fox et al 2007; 2015), meaning that colonies/populations are still being lost. With only two major populations in England, loss of either would have a huge impact, especially if those populations are found to be unique in behaviour or taxonomy.

With this in mind, landscape-scale conservation efforts are important to preserve the species by ensuring connectivity (Hanski & Gaggiotti 2004) and habitat quality (Slamova et al. 2013). Attention should be paid to the requirements of the English populations, which are known to use a different larval food plant than their Scottish counterparts; *Sesleria caerulea* (Oates 1995) rather than *Molinia caerulea* (Kinnear & Kirkland 2000).

It is generally accepted that the *E. aethiops* is divided into two separate subspecies; *E. aethiops aethops* and *E. athiops caledonia* (Figure 1.2). This is largely due to observations of morphological differences documented in 1777 by Esper and more fully described by Verity in 1922. The *aethiops* subspecies is described as being restricted to England and north-east Scotland, while the *caledonia* ssp covers the rest of Scotland. It is thought to be smaller with a narrower body and less distinct underwing markings (Thompson 1980)

Research has historically assumed the subspecies level distinction when studying the butterfly (Warren 1937; Blackie 1948; Thomson 1980; Newland 2012), although it has come under scrutiny in the last decade (Kirkland 2012) due to lack of evidence. Genetic analysis also found no indication of subspecies variation in the proposed locations (Gunson 2016; Iversen 2013).

1.6 Study aims

This study aims to advise future management and translocation efforts of *E. aethiops* in England by determining the genetic state and structure of the remaining English populations and comparing the connected mainland Scottish and isolated Scottish island populations. This study also investigates the flight capabilities and dispersal behaviour of *E. aethiops* as an additional indicator of gene flow between patches while also generating an up-to date population estimate at the Smardale Gill site.

The specific aims of the study were as follows:

1- To assess the level of genetic diversity in English and Scottish populations. This will allow identification of potential inbreeding in isolated populations, indicated by low heterozygosity, thereby marking those areas as priorities for management. This will also indicate historic bottleneck or founder events and will allow any future translocations to select highly diverse populations for individual removal.

2- To investigate the level of genetic differentiation between populations across Britain to give an historic view of their separation. Understanding if populations are significantly differentiated will allow the selection of a less differentiated source for the supplementation of a struggling population to increase breeding compatibility. If the English populations are differentiated from the Scottish, it may also mean that they have evolved to become warm-adapted and would be identified as the only option for southern reintroductions. In addition, this analysis will allow to establish whether the small populations at Crosby Garrett and Bastow Wood are true populations which have survived the range retraction or are unauthorised reintroductions and, if so, the source of the translocated individuals.

3- To measure, at a local scale, the genetic relatedness among individuals from different habitat patches and alongside with a mark-recapture study, to determine the level of connectivity within the Smardale Gill metapopulation. The study also aims to provide an overview of movement and gene flow within the reserve.

4- Finally, this project aims to develop a repeatable methodology for extracting useable DNA from a single leg. Previous studies have all used larger amounts of tissue which required the removal and euthanisation of individuals (Harper 2011; Iversen 2013; Gunson 2016) and the creation of a non-lethal procedure would allow much smaller populations to be sampled without decreasing numbers. It may also be beneficial for future genetic analysis on other butterfly species where only a very small amount of tissue is available or where non-lethal methods are preferable.

2. Methods

2.1. Study species

E. aethiops is one of the UK's latest emerging butterflies, spending the majority of its life as a caterpillar, then emerging in late July and flying until early September (Figure 2.1). The species is univoltine and will mate shortly after emerging.

Figure 2.1. Life cycle of *Erebia aethiops* in Britain (Butterfly-conservation.org 11/11/18).

As a specialist northern species, a main threat to *E. aethiops* is potentially climate change. The species is limited by high temperatures and spends the hottest part of the day in the shade (Slamova et al. 2011), meaning that habitat destruction is also a threat.

E. aethiops favours limestone grassland and woodland edges or clearings (Asher et al. 2001). The adults use a wide range of nectar sources, but the larval food plant is limited to purple moor grass (*Molinia caerulea*) in Scotland and blue moor grass (*Sesleria caerulea*) in England (Fox et al. 2006).

2.2. Study sites

Specimens for genetic analysis were collected from populations across the UK distribution of the species (Table 2.1, Figure 2.3). In England, only two natural populations of *E. aethiops* have persisted to the present day; the remainder of what was likely a continuous range following the last ice age and a more fragmented range from the early to mid-20th century (Thomas 2010). The two natural populations are located at Smardale Gill and Arnside Knott nature reserves, both in Cumbria (Figure 2.2).

A small population has been reported in recent years on a roadside verge (Patch 8 in Figure 2.5) and has been included in the Smardale Gill site for genetic analysis at a geographic scale due to the proximity to that site (<2km). In addition, specimens were

collected from a small population at Crosby Garrett in Cumbria of unknown origin and another small population believed to be the result of unauthorised release at Bastow Wood in Yorkshire (Figure 2.2). The Scottish specimens were collected two years previously from populations on the mainland and the Isles of Skye, Mull and Arran (see Gunson 2016 for details and Figure 2.2 for locations)

Table 2.1. *Erebia aethiops* sample collection sites, date of collection and number of individuals collected.

Site	Region	Grid ref.	Year	Sample size
Insh Marshes	Scottish Mainland	NH771003	2015	5
Craigower	Scottish Mainland	NN927605	2015	5
Tomnavoulin	Scottish Mainland	NJ211261	2015	5
Skye	Islands	NG414384	2015	5
Mull	Islands	NM728363	2015	5
Arran	Islands	NR950363	2015	5
Smardale Gill	England	NY726068	2017	42
Arnside Knott	England	SD456774	2017	6
Crosby Garrett	England	NY728094	2017	5
Bastow Wood	England	SD991657	2017	7

5 was selected as a minimum sample size due to permission restrictions from landowners and management organisations and is comparable to minimum sizes used in AFLP analysis in previous studies investigating differentiation (Coart et al. 2002; Kingston & Rosel 2004).

Figure 2.2. *Erebia aethiops* collection sites for samples included in this study. Created with Google Maps.

For the genetic analysis at local scale, specimens were collected from different habitat patches (six patches) within the largest English site, Smardale Gill. Patches correspond with those used for the mark-release-recapture study (see Figure 2.2 and specific details for each patch in Mark-Release-Recapture section).

Number of collected individuals from each site (or patch for Smardale Gill site) varied depending on the population size at the site and the observed individuals during the collection period with, a minimum of 5 individuals from the smallest populations (Table 2.1). All collected individuals were allocated a code based on their origin (Appendix 1).

5 was chosen as the minimum sample number to match the numbers taken from Scotland in previous years (Gunson 2016) and after consulting the literature. AFLP analysis has been successfuly performed to give a standard error of 10% of the population diversity (Singh et al. 2006).

2.3. Specimen collection procedure

Permission to remove individuals from the sites was obtained from landowners and reserve management. As Arnside Knott and Smardale Gill are SSSIs, permission was also obtained from Natural England.

Adult *E. aethiops* fly from late July to early September (Asher et al. 2001), so no collection was done before late August. Males in very poor condition were targeted wherever possible to avoid removing egg-carrying females and impacting the effective population. Individuals which were found already dead were collected regardless of sex and condition.

In Arnside Knott, Crosby Garret and Bastow Wood, there were not enough males to remove the required number (at least 5) without damaging the population. In these cases, a leg was removed from a female (Appendix 1).

Capture and in-field euthanisation followed protocols recommended by Feinstein (2004) and Prendini (2002), which also match the methods used to collect the Scottish samples (Gunson 2016). Capture was performed with a net and euthanisation was via a quick pinch to the abdomen. Other methods were considered but were unsuitable. Euthanisation and storage in ethyl acetate is an accepted method but it has been found to reduce DNA yield (Iversen 2013; Feinstein 2004). Freezing, while a recommended euthanisation method which has been shown to effectively preserve DNA (Prendini 2002) was discounted due to in-field restrictions.

All samples were stored separately in their own paper packets or test tubes and hands were cleaned or gloves changed between collections to avoid cross-contamination. Samples were refrigerated on the day of collection and were frozen at -20 for long-term storage within several days.

2.4 Molecular technology in conservation

A popular method of genetic analysis is Amplified Fragment Length Polymorphism (AFLP); a PCR based process developed which uses primers to selectively target restriction fragments for amplification (Zabeau & Vos 2000). Data resulting from AFLP-PCR are actually scored as presence/absence polymorphisms rather than length polymorphisms as the name suggests (Vos et al. 1995).

Unlike microsatellite analysis, AFLP-PCR requires no prior genetic knowledge about the target species. The technique has increased in popularity due to their ease of use and high replicability (Vuylsteke et al. 2007).

AFLP is commonly used in population genetics in general (Mueller & Wolfenbarger 1999) and in butterfly studies specifically (Jiggins et al. 2005; Brattström et al. 2010), where it has been shown to give concordant results to microsatellite analysis (Smee et

al. 2013). It has also picked up species-level differentiation where mitochondrial analysis of a single locus did not (Gompert et al. 2006), highlighting the advantages of multi-locus techniques in conservation genetics.

The protocol is relatively easy to modify compared to other multi-locus sampling techniques (Bensch & Åkesson 2005) and AFLP optimisation has already been done for the study species, *Erebia aethiops* by Harper (2011). The existence of a previous protocol facilitated the lab work and also allowed comparison of results presented in this thesis with those reported by Harper (2011).

Another major benefit of AFLP is that it has been shown to work on relatively low DNA concentration yields (Janssen et al. 1996; Harper 2011) meaning very small amounts of tissue could potentially be used.

Previous studies of *E. aethiops* have used the thorax to successfully extract required amounts of DNA for MtDNA barcoding (Gunson 2016; Iversen 2013) and AFLP-PCR (Harper 2011) but this is a lethal sampling method which requires the permanent removal of individuals from the population. Genetic studies on large insects have used leg DNA in AFLP analysis (Zhang et al. 1995; Kethidi et al. 2003) or wing (Keyghobadi et al. 2009; Crawford et al. 2011).

Non-lethal sampling includes removal of an entire leg or part of a wing and has been shown to have no significant effect on a butterfly's behaviour, longevity or survival (Hamm et al. 2010; Marschalek et al. 2013) making it an option for protected or scarce species.

2.5. DNA extraction and AFLP-PCR

2.4.1. DNA extraction

For individuals which were euthanised and collected whole, DNA was extracted from half the thorax with the other half being retained in case of failure. Extraction was performed using a QIAGEN DNeasy Blood and Tissue kit and followed the suggested protocols (Appendix 2) with some modifications (Iversen 2013; Gunson 2016). Incubation temperature was increased from 56°C to 57°C and incubation time from 1 hour to >5 hours to account for the slower rate of cellular breakdown in insect tissue.

Further modifications were needed to successfully extract DNA from a single leg in the required concentration for AFLP-PCR (10ng DNA per µl suspension buffer). Tests were performed, and a working methodology was generated. This followed the thorax extraction protocol with some modifications:

- The leg was processed while still frozen to make the tissue more brittle and facilitate cellular breakdown.
- Rather than cutting, tissue was ground in a pestle and mortar with 100 µl ATL buffer, with a further 100 µl added to 'rinse' the mortar to ensure all tissue was collected via pipette.
- A second vortexing stage was added approximately half way through incubation, with a minimum of 5 hours incubation beforehand.
- Incubation time was increased to >8 hours, ideally overnight.
- All remaining tissue precipitate was added to the spin column prior to centrifuging.
- Final elution volume of AE buffer was reduced by ¾ from 200 µl to 50 µl. Higher volumes were tested but 50 µl is the maximum volume to reliably achieve the required concentration.

All extractions were tested via electrophoresis through agarose gels (Figure 2.3). Leg DNA did not consistently show up following UV gel imaging due to lower concentrations (an average of 11ng/µl compared to >300ng/µl from the thorax) so the success of the extraction was confirmed using a nanodrop spectrophotometer. Thorax DNA was also assessed, and all concentrations were noted for pre-AFLP dilution.

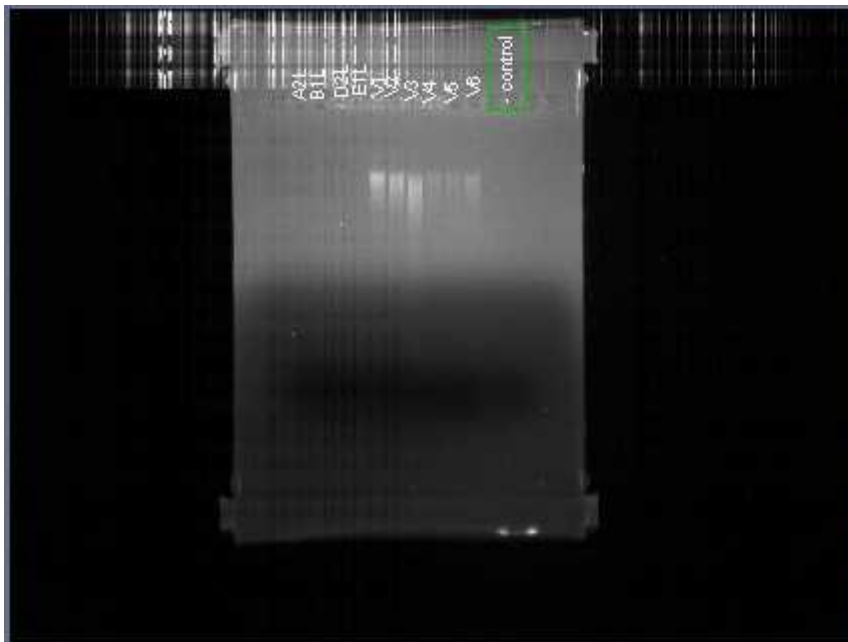

Figure 2.3. Agarose gel following electrophoresis to assess the success of DNA extraction. Smears indicate DNA presence. Columns with no smear are leg extractions which were later found to be successful using a nanodrop spectrophotometer.

2.5.2. AFLP-PCR

All samples were diluted to 10ng/µl prior to amplification except those which were at a lower concentration (6 total). The base protocol was modified from Paun & Schonswetter (2012) (Appendix 3) with restriction and ligation phases combined. The DNA/H_2O dilution step was omitted for lower yield DNA (<10ng/µl) prior to combination with the restriction-ligation master mix and incubation to give a total of 55ng DNA per sample for amplification. Only the required amount of DNA was diluted with the remainder retained at the original concentration and frozen at -20°C for future use.

EcoRI and MseI restriction endonucleases were used along with corresponding forward and reverse adapters, which were mixed and heated to 95ºC for 5 minutes and cooled prior to use to allow annealing of the sequences:
EcoRI (A1) CTCGTAGACTGCGTACC & EcoRI (A2) AATTGGTACGCAGTCTAC;
MseI (A1) GACGATGAGTCCTGAG & MseI (A2) TACTCAGGACTCAT

Alterations were made to reagent volumes depending on base concentration and the thermal cycle was set to 37°C for two hours and 17°C overnight for a minimum of 8 hours.

Immediately following incubation, 190 µl H_2O was added to halt the reaction. For <10ng/µl samples, dilution was reduced to increase concentration, with the lowest mix being a 3.8 ng/µl solution with 70µl H_2O added. These dilutions were used in the pre-selective stage.

Each sample underwent pre-selection twice with two different pairs of pre-selective PCR primers:

EcoRI primer (A) GACTGCGTACCAATTCT & MseI primer GATGAGTCCTGAGTAAC;
EcoRI primer (B) GACTGCGTACCAATTCA & MseI primer GATGAGTCCTGAGTAAC

2µl of restriction-ligation reaction product was used for each pre-selective pair and were cycled at:

One cycle of 72°C – 2 min

20 cycles of:

94°C – 20 s

56°C – 30 s

72°C – 1 min

One cycle of 60°C – 15 min

To generate *E. aethiops* AFLP markers during selective PCR, each sample underwent selection 3 times with different primer pairs added to their corresponding pre-selection product:
EcoRI-TCT & MseI-CAA
EcoRI-TGA & MseI-CTG
EcoRI-ATC & MseI-CTG

Fluorescent dyes were added to each pair (HEX, FAM, ATTO) as labels for future fingerprinting via capillary electrophoresis and each sample was cycled at:

One cycle of 94°C – 2 min

9 cycles of:
    94°C – 30 s
    65°C -1°C /cycle – 30 s
    72°C – 2 min

23 cycles of:
    94°C – 30 s
    56°C – 30 s
    72°C – 2 min

One cycle of 72°C – 10 min

AFLP products were sent in 96-well plates to DBS Genomics for fragment analysis using an Applied Biosystems 3730 DNA Analyser with a DS-30 filter set ROX500 size standard and with ATTO dye replacing the usual NED due to supplier availability. All 3 products for each sample were multiplexed in a single well to achieve higher throughput. To test in-house accuracy, 20 samples were amplified twice on separate occasions and the results were checked to ensure they corresponded.

Following AFLP-PCR, tests were performed to determine the optimal ATTO:HEX:FAM multiplexing ratio and PCR product:H_2O dilutions for fingerprinting with negative controls included for each combination. Simple 1:1:1 ratios and undiluted product were selected and the fragment detection was recorded as peak heights in .fsa files for analysis.

2.6. Population size and dispersal at a local scale

2.6.1. Study site

Smardale Gill Nature Reserve is located in Cumbria, near the town of Kirby-Stephen and is bisected by Scandal Beck, which flows to the River Eden. A disused viaduct makes up part of a footpath and crosses the river (Figure 2.4). There is also a disused lime kiln which is built into a slate-topped hill.

The 49-hectare site is made up of unimproved limestone grassland and woodland, with cattle grazing in contained areas. The terrain is steeply sloped, with rocky

outcrops and wooded patches. The primary vegetation is mixed grasses and herbaceous plants with several notable species such as bloody cranesbill, fragrant and greater butterfly orchids, rock rose and horseshoe vetch.

Blue moor grass (*Sesleria caerulea*), *E. aethiops*' larval food plant, is common throughout the reserve, with the largest patch present on the East side of the viaduct (Figure 2.4). Other potential suitable habitat patches were identified based on records of the species from transect surveys (UKBMS) and consultation with Butterfly Conservation and Smardale Gill Wildlife Trust staff. These were narrowed down to areas containing the larval food plant with a visible barrier (such as a road, a wider band of tall trees or a grazed field) separating them (Figure 2.5). One additional patch was added during the MRR study (Patch 5 in Figure 2.5) due to sightings of individuals in an area with no visible larval food plant.

Figure 2.4. Picture of Smardale Gill Nature reserve (photo source: Rosa Menendez).

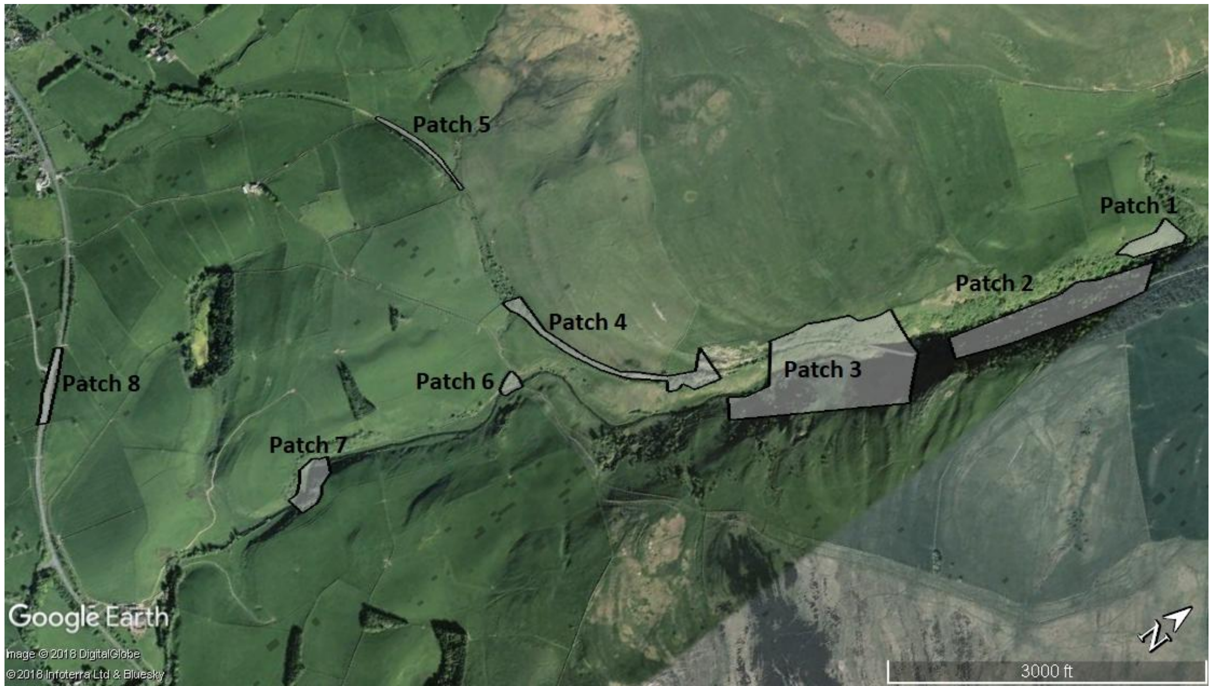

Figure 2.5. Location of habitat patches occupied by *Erebia aethiops* in Smardale Gill and used in the mark-release-recapture study (photo source: Google Earth).

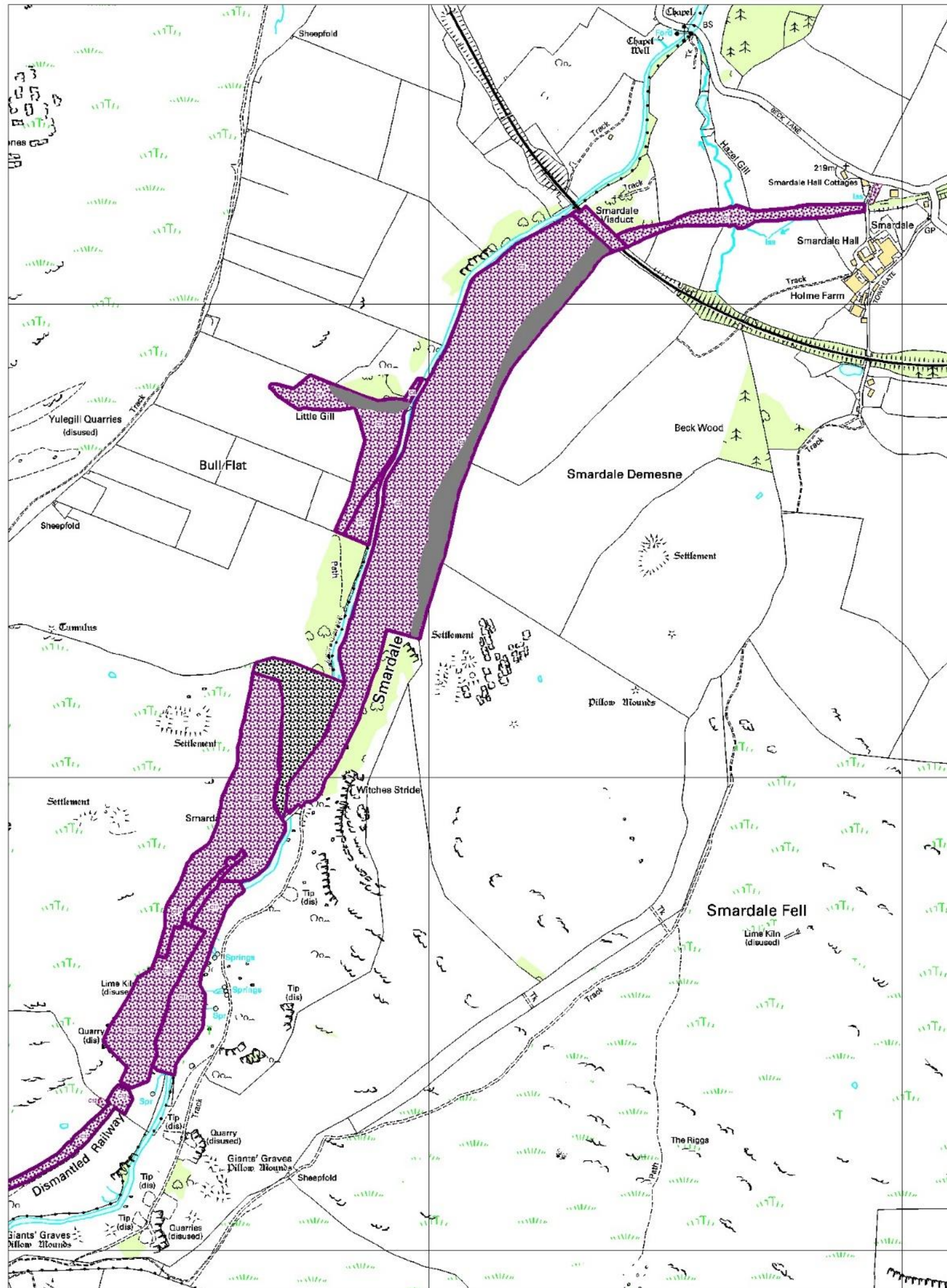

Figure 2.6.a. Smardale Gill reserve - north. Bold line marks reserve boundary. Shaded area indicates areas walked/surveyed to determine presence of potential habitat. Filled grey area indicates areas which were inaccessible due to vegetation or slope gradient.

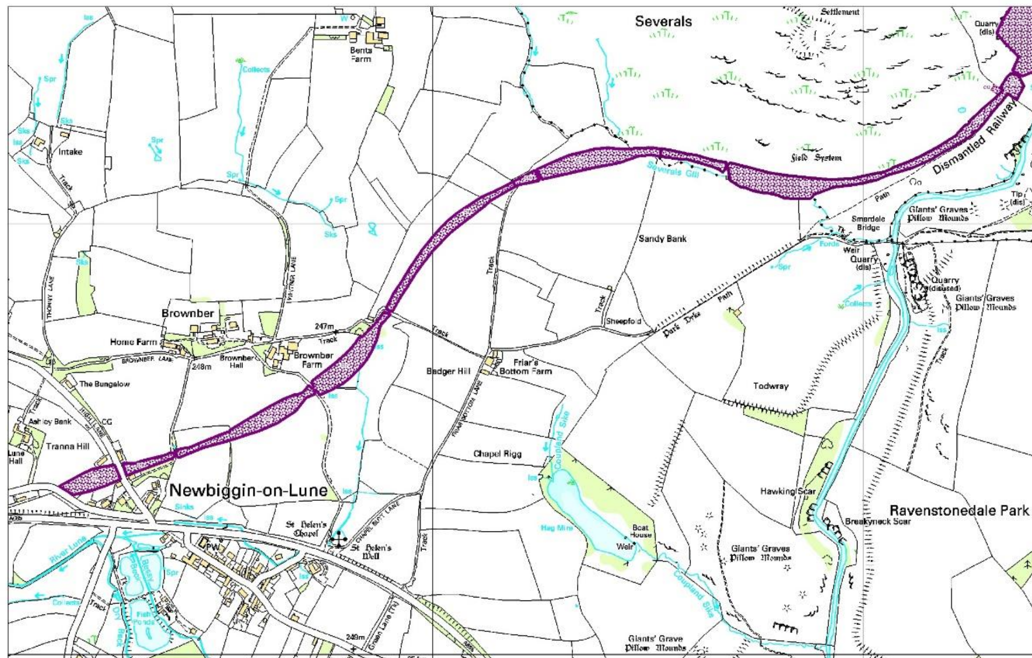

Figure 2.6.b. Smardale Gill reserve - south. Bold line marks reserve boundary. Shaded area indicates areas walked/surveyed to determine presence of potential habitat.

2.6.2. Mark, Release and Recapture study

A mark, release and recapture (MRR) study was undertaken in Smardale Gill Nature Reserve to determine the population size and dispersal ability of *E. aethiops*.

Patches are defined as areas containing suitable *E. aethiops* habitat, separated by a boundary, such as unsuitable habitat or structures. Initially, surveying was restricted only to areas which contained the larval food plant but an additional patch was included during the first MRR visit after butterflies were seen in Patch 5.

MRR was carried out from 25th of July to 6th of September 2017 for a total of 20 days surveyed. *E. aethiops* is active during warm, sunny periods (Slamova at al. 2011), so only days with favourable weather were included. Each study patch was visited on a rotational basis at varying times of day with an equal amount of time spent at each patch with adjustments being made for patch size. For example, a patch which was twice the size of another was allocated twice the survey time. Each patch was visited at least once every two days by one of two researchers and the same route was taken along all safely accessible areas.

Every unmarked butterfly encountered (after in-hand examination) was marked on the underside of the second pair of wings with a number, using an indelible fine-line marker pen (Figure 2.7) before being released in its original capture location. Tens

and singles digits were marked on the left wing while hundreds and thousands were marked on the right wing. This was selected as the most appropriate method after in-lab testing using similarly size and colour pattern species (*Maniola jurtina* and *Aphantopus hyperantus*).

Figure 2.7. Mating male (number 997) and female (number 1823) of *Erebia Aethiops* (photo source: Rosa Menendez).

Following capture, a note was made of the butterfly's number (if previously marked), previous capture (Y/N), time, date, patch, GPS coordinates, sex, condition (based on wing wear on a scale of 0-2 with 2 being perfect condition) and behaviour. Recapture events were counted if they were three hours apart or were in a different patch than the previous capture.

2.7. Data analysis

2.7.1. AFLP-PCR

Files containing fragment analyses (.fsa) were loaded into PeakScanner (Applied Biosystems 2006) for visualisation. Custom parameters were used to create light

smoothing of the electropherogram where background noise may have generated false secondary peaks. The upper fluorescence threshold was set to the mid-upper limit of technical noise at 50 Relative Fluorescent Units (RFU), with the intention of filtering out missed false-presences during binning.

A table of peak locations and size was created and exported as a text-tabulated file. Red dye peaks from the ROX500 size standard were not included.

The table was converted to a presence/absence binary matrix using the RawGeno automated scoring R package (Arrigo et al. 2012) with primer pairs converted separately, then merged.

Parameters were set to calculate 1%-99% quantiles of detected AFLP peaks and retain only individuals which fell within those bounds for all 3 primer combinations. Three samples were removed at this stage (CG2, C3, E1; Appendix 1) along with negative controls.

To allow very minor bp location differences to be disregarded and avoid over splitting peaks according to recommendations made by Holland et al. (2008), a maximal bin width of 2 bp was set. A minimal bin width of 1 bp was specified to avoid technical homoplasy (false assignment of multiple peaks from an individual into the same bin). In total, 655 loci were retained in a binary matrix.

The binary matrix was entered into AFLP-SURV (Vekemans et al 2002) to estimate genetic diversity. Assuming Hardy-Weinberg genotypic proportions, a Bayesian method with non-uniform prior distribution of allele frequencies was selected to calculate allelic distribution for each population separately (Zhivotovsky 1999). This generated estimates for: Proportion of polymorphic loci at the 5% level, expected heterozygosity (He.), individual to individual relatedness coefficients (Rab) and Wright's fixation index (Fst) with 500 permutations.

2.7.2. Regional analysis

An ANOVA test was performed on the expected heterozygosity (He.) of the three geographic regions (England, Scottish mainland and Scottish islands) to determine whether there were significant differences in genetic diversity levels between regions.

Pairwise Fst estimates between all sites were used to infer interbreeding history and to test for significant differentiation between the populations. An AMOVA test with 999 permutations was conducted to determine levels of genetic differentiation among regions, among sites and within sites. The relationship between genetic distance and geographic distance was tested with a Mantel test using the *GenAlEx* software (Peakall and Smouse 2006, 2012). To visualise overall genetic structure among the

study E. aethiops populations, a principal component analysis (PCA) was conducted using the *Adegenet* package in R (Jombart 2008).

Relatedness between sites was calculated as the average Rab for individuals in a site-site pairwise comparison. This allowed to estimate the most likely point of origin for the Bastow Wood and Crosby Garett populations.

2.7.3 Local analysis

Pairwise Fst estimates between patches within the Smardale Gill site were used to infer interbreeding history, separation history and gene flow between patches. A Mantel test, conducted in *GenALEx* was used to assess correlation between genetic distance and geographic distance for all patches. Observed Fst was also used to determine whether there was significant differentiation between the patches.

2.7.4. MRR analysis

Estimates of population size were generated using the *RCapture* package in R (Baillargeon et al. 2007) for both overall and patch-specific estimates.

Estimates were generated at intervals of 2 survey days to show number of individuals present in the population at specific times as well as identifying the peak of the flight season.

Log-linear models assuming equal and unconstrained capture probabilities for open populations were fitted and the Akaike's Information Criterion (AIC) values were used to identify the best model (the one with a higher AIC, with a difference of >2 was discarded). The model with equal capture probability was the best model for all individual patches. For the overall reserve estimate, however, both models had similar AIC values and the equal probability model was used as it produced lower standard errors and for consistency with the estimates for individual patches.

To assess dispersal, the maximum distance moved by an individual between recaptures was calculated for all recaptured individuals (rounded down to the nearest 10m to compensate for GPS inaccuracy).

A Mann-Whitney U test was used to determine significance difference in the distances moved by males and females.

3. Results

3.1 Genetic analysis

A total of 86 individuals from 10 populations were genotyped and were represented by 655 loci with 633 (96.6%) segregating fragments which ranged in size from 50-485 base pairs.

3.1.1 Genetic diversity and differentiation across Britain

Expected heterozygosity (He) of *E. aethiops* populations across Britain (Table 3.1) ranged from 0.146 to 0.219 (mean = 0.190, S.E.= 0.007), with a polymorphic loci proportion range of 55.6-37.4% (mean = 46.99%, S.E.= 1.94).

Diversity levels differ slightly between the three sampled regions, particularly England and Scotland (Figure 3.1), however an ANOVA test showed marginally no significant difference (F = 3.589, d.f.= 2, p=0.085). Among the English populations Crosby Garrett showed the highest genetic diversity (He), while Smardale Gill showed the lowest, with no overlapping standard errors. For the Scottish populations the Island of Mull showed the highest genetic diversity while Insh Marshes showed the lowest of any population (Table 3.1).

Table 3.1. Genetic diversity (He. and proportion of polymorphic loci) of *Erebia aethiops* populations across Britain. Samples from English (E), Scottish mainland (S) and Scottish island (I) populations.

Population	N	Polymorphic loci (%)	Expected Heterozygosity (He)	S.E. (He)
Smardale Gill (E)	42	55.6	0.182	0.008
Crosby Garrett (E)	4	48.4	0.219	0.008
Arnside Knott (E)	6	55.1	0.212	0.007
Bastow Wood (E)	7	51.6	0.201	0.008
Insh Marshes (S)	5	37.4	0.146	0.007
Craigower (S)	5	46.1	0.186	0.007
Tomnvoulin (S)	4	39.5	0.175	0.008
Skye (I)	4	42.0	0.180	0.007
Mull (I)	4	48.1	0.210	0.008
Arran (I)	5	46.1	0.189	0.008

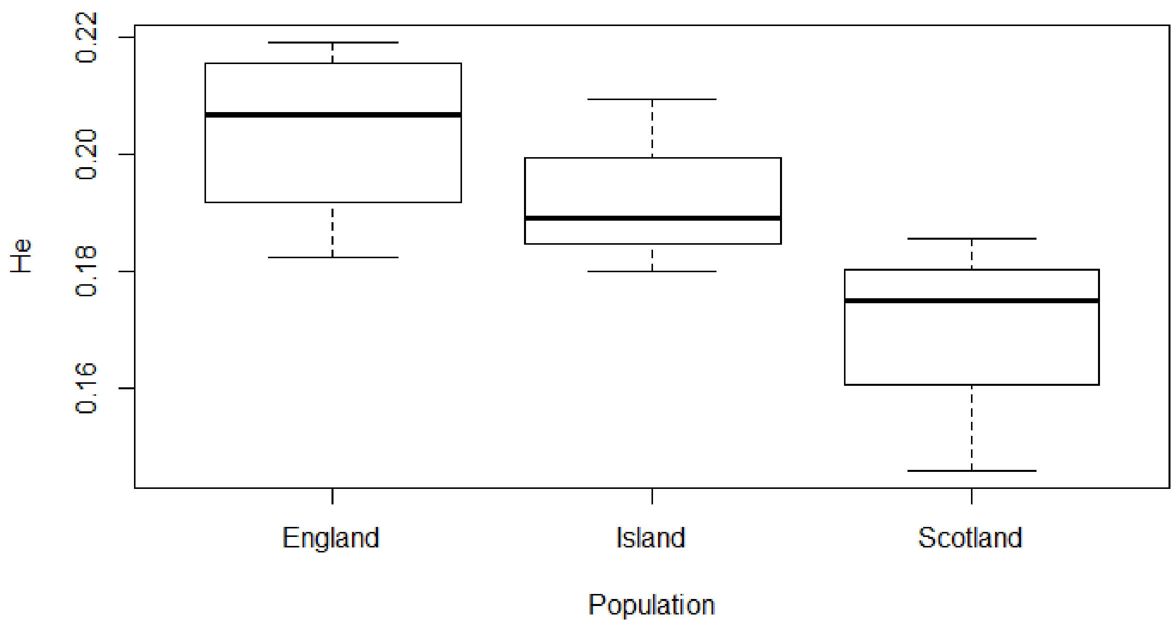

Figure 3.1. Expected Heterozygosity (He) in English, Scottish mainland and Scottish island populations of *Erebia aethiops*. Boxplots displaying the median, the first and third quartile and the maximum and minimum values.

Table 3.2 shows Fst pairwise values between populations as a measure of genetic distance due to interbreeding. Discounting like/like comparisons, the most genetically connected populations are the Scottish mainland populations, with the islands showing the highest levels of isolation. Differentiation levels are assessed using Hartl & Clark's (1997) scale:

0 = No differentiation

<0.05 = Little differentiation

0.05 – 0.15 = Moderate differentiation

0.15 – 0.25 = Great differentiation

The overall observed Fst value, obtained via 500 permutations and tested against random permuting of individuals within-population was 0.085 (S.E. 0.018). This value is much higher that the higher and lower 95% limits (0.020 and 0.013 Fst value under the null hypothesis of no differentiation, respectively), meaning the populations are more genetically differentiated than a random assemblage. This result is also supported by a calculated p value of less than 0.001 which is used to assess the likelihood of rejecting the null hypothesis of a random assemblage.

Moreover, there were significant differences in genetic divergence among regions and populations (Table 3.3). Differences among regions significantly explained 10% of the genetic variation with an additional 6 % of variation explained by differences among

populations, the remained variation (84%) was explained by differences between individuals within populations.

Table 3.2. Pairwise Fst values between all sampled populations in Britain. A value of 0 indicates total panmixis, while higher values indicate reduced mixing. * indicates moderate or higher differentiation, X indicates no or little differentiation. **Bold** values indicate great differentiation.

	Smardale Gill	Crosby Garrett	Arnside Knott	Bastow Wood	Insh Marshe	Craigower	Tomnavoulin	Skye	Mull	Arran
Arran	*	*	*	*	*	*	*	*	*	0
Mull	*	*	*	*	*	*	*		0	0.072
Skye	*	*	*	*	X	X	X	0	0.126	0.141
Tomnavoulin	*	*	*	*	X	X	0	0.021	0.094	0.144
Craigower	*	*	*	*	X	0	0	0.013	0.079	0.085
Insh Marshes	*	*	*	*	0	0.001	0	0.041	0.105	**0.160**
Bastow Wood	X	X	X	0	0.102	0.067	0.075	0.094	0.097	0.120
Arnside Knott	*	*	0	0.034	**0.163**	0.132	0.132	**0.164**	0.121	**0.164**
Crosby Garrett	X	0	0.080	0.036	0.076	0.053	0.055	0.061	0.076	0.091
Smardale Gill	0	0.029	0.063	0.005	0.092	0.067	0.066	0.090	0.107	**0.126**

Table 3.3. AMOVA results (based on 999 permutations) for genetic differentiation of *Erebia aethiops* individuals with variation sources nested among regions, among populations within regions, and within populations.

Variation source	Explained variation (%)	F-value	d.f.	p-value
Among regions	10	0.101	2	<0.001
Among populations	6	0.062	7	<0.001
Within populations	84	0.157	76	<0.001

The Principle Component Analysis organised individuals into clusters based on genetic similarity (Figure 3.2) to allow visualisation of separation and overlap between populations. The first two PCA components explained 11.8 % and 6.8 % of the variation in genetic composition among populations. All sampled populations showed some overlap with at least one other, with clustering evident between the English and Scottish populations, but separation from the islands, particularly Arran.

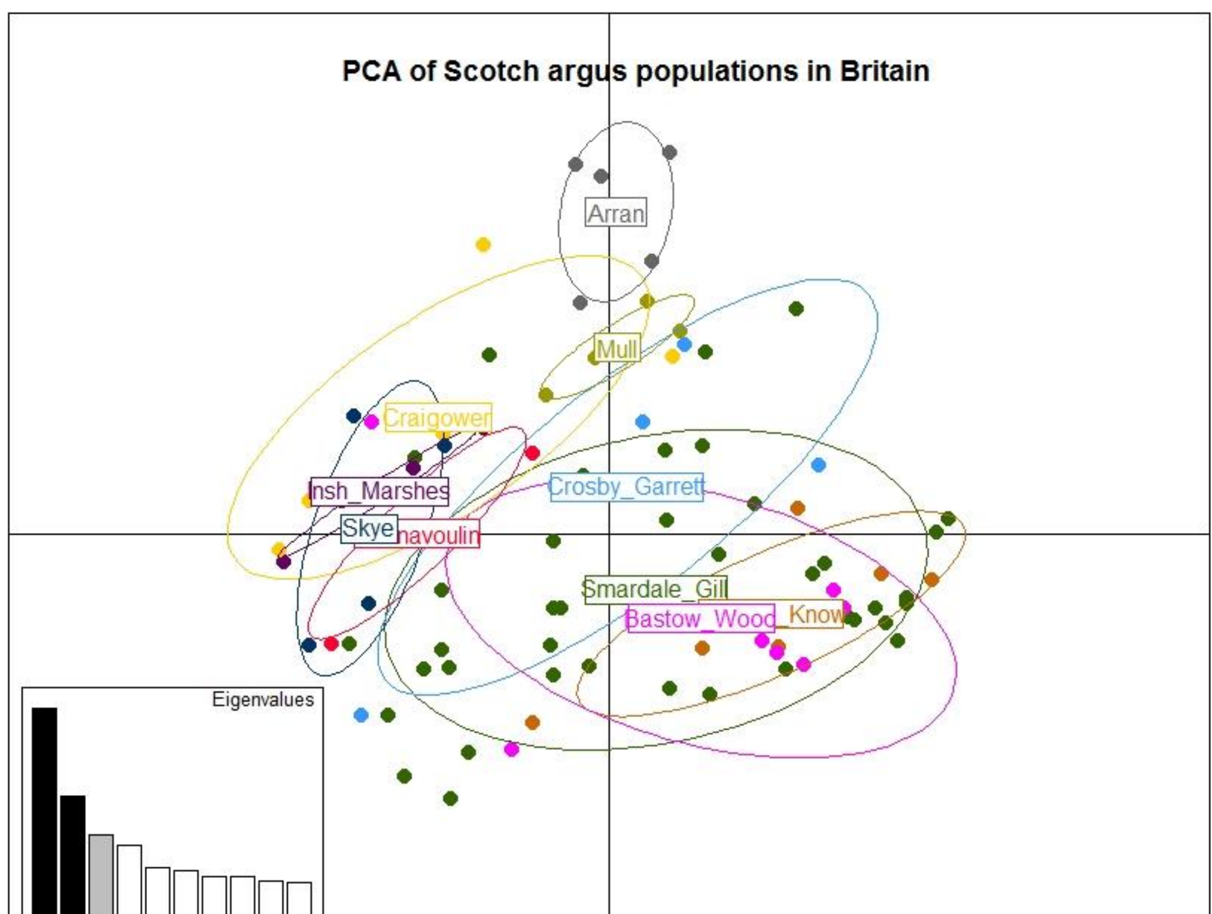

Figure 3.2. Principal Component analysis (PCA) for *Erebia aethiops* individuals from different populations across Britain (data from AFLP-PCR of presence/absence of 655 loci). The PCA was implemented in *Adegenet* package in R. PC1 is presented in x-axis and PC2 in y-axis, inertia ellipses are presented for each population.

Relatedness (rab) as an average of the relatedness coefficients between all individuals (Table 3.4) is an historic measure of genetic relationship. A higher relatedness value = lower genetic distance (1-rab) and a more recent divergence from a common ancestor and/or geographic separation.

Relatedness is a primarily comparative measure, so relatedness of a population to itself gives a baseline for comparison. The highest levels of relatedness, excluding like/like comparisons, are found between Smardale Gill and Bastow wood (0.281). This also shows that the most likely origin of Bastow Wood population, the one presumably resulting from an unauthorised reintroduction, is Smardale Gill. The lowest value/greatest genetic distance is found between Arnside Knott and the Isle of Skye (-0.014). Scottish mainland populations were combined in this calculation as there is panmixis between them, meaning they should be treated as a single possible point of origin.

Table 3.4. Historic pairwise relatedness between British *Erebia aethiops* populations.

	Smardale Gill	Crosby Garrett	Arnside Knott	Bastow Wood	Skye	Mull	Arran	Scottish Mainland
Smardale Gill	0.251							
Crosby Garrett	0.145	0.149						
Arnside Knott	0.181	0.067	0.265					
Bastow Wood	0.281	0.164	0.244	0.346				
Skye	0.088	0.026	-0.014	0.102	0.159			
Mull	0.124	0.092	0.075	0.158	0.079	0.269		
Arran	0.102	0.081	0.039	0.123	0.043	0.160	0.292	
Scottish Mainland	0.220	0.147	0.131	0.243	0.216	0.221	0.162	N/A

A Mantel test of 999 permutations comparing pair-wise genetic distance (Fst) to geographic distance among populations, showed a slight positive significant correlation between the two variables (Figure 3.3, $r = 0.284$, $p = 0.033$).

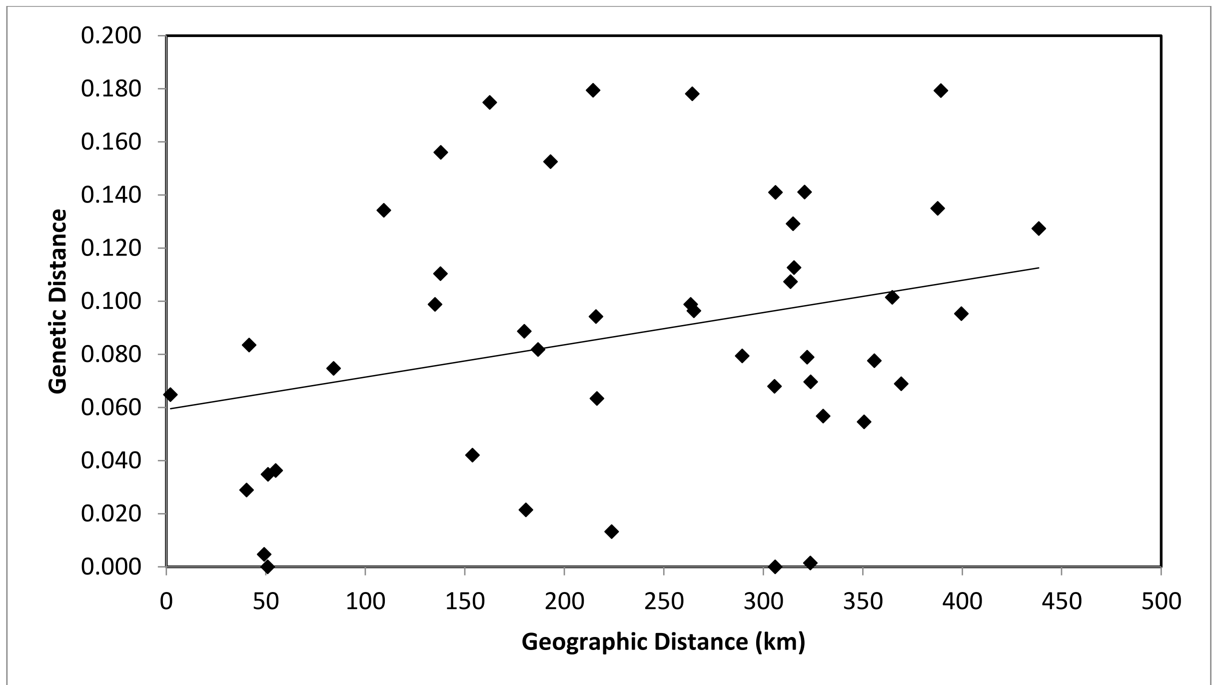

Figure 3.3. Relationship between pairwise genetic (Fst) and geographic distance of British *Erebia aethiops* populations.

3.1.2 Genetic diversity and differentiation in Smardale Gill Nature Reserve

Expected heterozygosity (He) of *E. aethiops* in Smardale Gill ranged from 0.151 and 0.214 (mean = 0.184, S.E. = 0.007) with a polymorphic loci proportion range of 36.5%-58% (mean = 46.88%, S.E. = 3.3) (Table 3.5).

Table 3.5. Genetic diversity of *Erebia aethiops* populations (He. and proportion of polymorphic loci) from six individual habitat patches within Smardale Gill Nature Reserve.

Patch	N	Polymorphic loci (%)	Expected Heterozygosity (He)	S.E. (He)
Patch 1	5	36.5	0.151	0.007
Patch 2	5	39.1	0.155	0.007
Patch 3	15	58.0	0.214	0.007
Patch 4	5	48.5	0.201	0.007
Patch 7	6	52.5	0.203	0.007
Patch 8	6	46.7	0.177	0.007

Table 3.6 shows pairwise Fst values between patches as a measure of genetic distance due to interbreeding. Discounting like/like comparisons, the most genetically connected patches are 4 and 7, but every patch shows a measure of connectivity to at least one other, with patch 3 showing the lowest level of mixing.

The observed Fst value for Smardale Gill only (comparing populations in different habitat patches) was 0.044 (SE. 0.002), which is higher than the lower and upper 95% limits (-0.021 and 0.014, respectively) and had a high-P value less than 0.001. This means that, although the differentiation is less than that between geographically separated populations (Fst = 0.085 among populations), there is some degree of genetic differentiation among patches that is significantly different than expected by chance.

Table 3.6. Pairwise Fst values between populations from six individual habitat patches within Smardale Gill Nature Reserve. A value of 0 indicates total panmixis, while higher values indicate reduced mixing. * indicates moderate or higher differentiation, X indicates no or little differentiation. No great differentiation observed.

	Patch 1	Patch 2	Patch 3	Patch4	Patch 7	Patch 8
Patch 1	0	X	*	X	*	X
Patch 2	0.016	0	*	X	*	*
Patch 3	0.129	0.103	0	X	X	*
Patch 4	0.006	0.027	0.032	0	X	X
Patch 7	0.081	0.054	0	0.002	0	X
Patch 8	0.045	0.064	0.055	0.005	0.029	0

3.2. Mark, release and -recapture at Smardale Gill Nature Reserve

A total of 1,697 individuals (1178 males and 519 females) were caught and marked, with 193 recaptured (153 males and 40 females) at least once for a total of 1,868 capture events.

3.2.1. Population estimates

The population of *E. aethiops* at Smardale Gill Nature reserve was estimated to be 7,869 individuals (± 688 S.E.) for the area as a whole. Population estimates for individual habitat patches vary from 24 individuals at the smaller patch to 9,747 individuals at largest patch, slightly higher than for the area as a whole, but the standard error for this estimate was very high (Table 3.7).

Table 3.7. Number of marked individuals, recapture events and population estimates for *Erebia aethiops* at Smardale Gill Nature Reserve. Population estimates, and S.E. are given for the entire flight period (2nd August – 6th September) for the area as a whole (Overall) and for each individual habitat patch (see Figure 3.5 for the location of each path). N/A indicates insufficient recaptures to generate a reliable patch-specific estimate.

Patch	Marked	Recapture events	Total events	Population estimate	S.E.
Patch 1	50	3	53	N/A	N/A
Patch 2	191	13	204	1367.6	432.9
Patch 3	1251	80	1331	9747.5	1292.5
Patch 4	78	22	100	179.5	33.2
Patch 5	14	6	20	24	7.3
Patch 6	13	1	14	N/A	N/A
Patch 7	61	13	74	176.1	47.4
Patch 8	39	33	72	56.5	8.6
Overall	1697	171	1868	7868.9	688.3

Figure 3.4 shows the changes in population size throughout the flight period at 2-day intervals for the whole nature reserve. The first individuals were seen on 2nd August, followed by a sharp increase to a peak in population size in mid-August and a progressive decline until the 6th September when the MRR ended. MRR results assume that there is an equal probability of capture each day and that populations are open to emigration/immigration and loss by death.

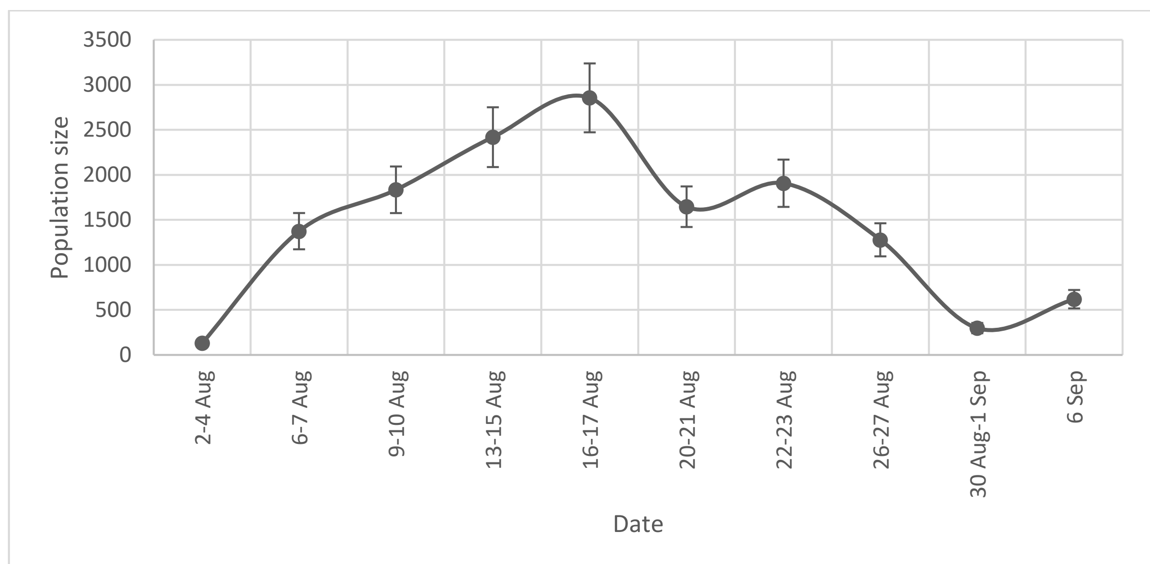

Figure 3.4. Changes in population estimates (±S.E.) of *Erebia aethiops* in Smardale Gill Nature Reserve throughout the 2017 flight period at 2-day intervals.

3.2.2 Movement

Of the 1,697 marked individuals, 232 movements (recaptures) in different days were recorded for 193 individuals. Of these, 13 were inter-patch movements, all completed by males (Figure 3.5). Most movement occurred between the largest central patch (patch 3) of the metapopulation and the surrounding patches and no movements were observed from and to the most isolated patches (patches 7 and 8).

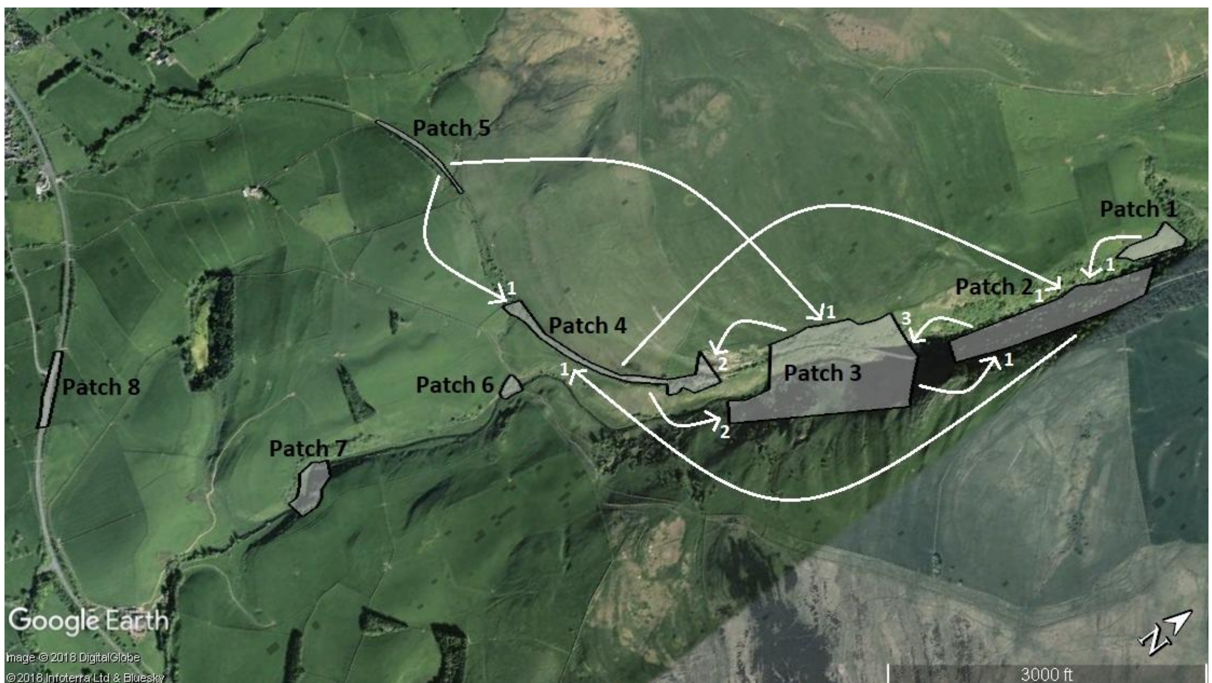

Figure 3.5. Movement between Smardale Gill patches. Lines indicate movement between patches and arrows indicate movement direction. Numbers above arrows indicate number of individuals that completed the movement.

The highest total distance travelled was 80m for females and 1,560m for males, with 4 males travelling ≥ 1,000m (Figure 3.5). However, most individuals moved short distances with 78% of males and 100% of females moving less than 100m. The mean distance travelled by females was 21.25m (± 3.97) and 110.5m (± 20.53) for males. A Mann-Whitney U test showed a significant difference between sexes ($w = 2030$, $p<0.001$).

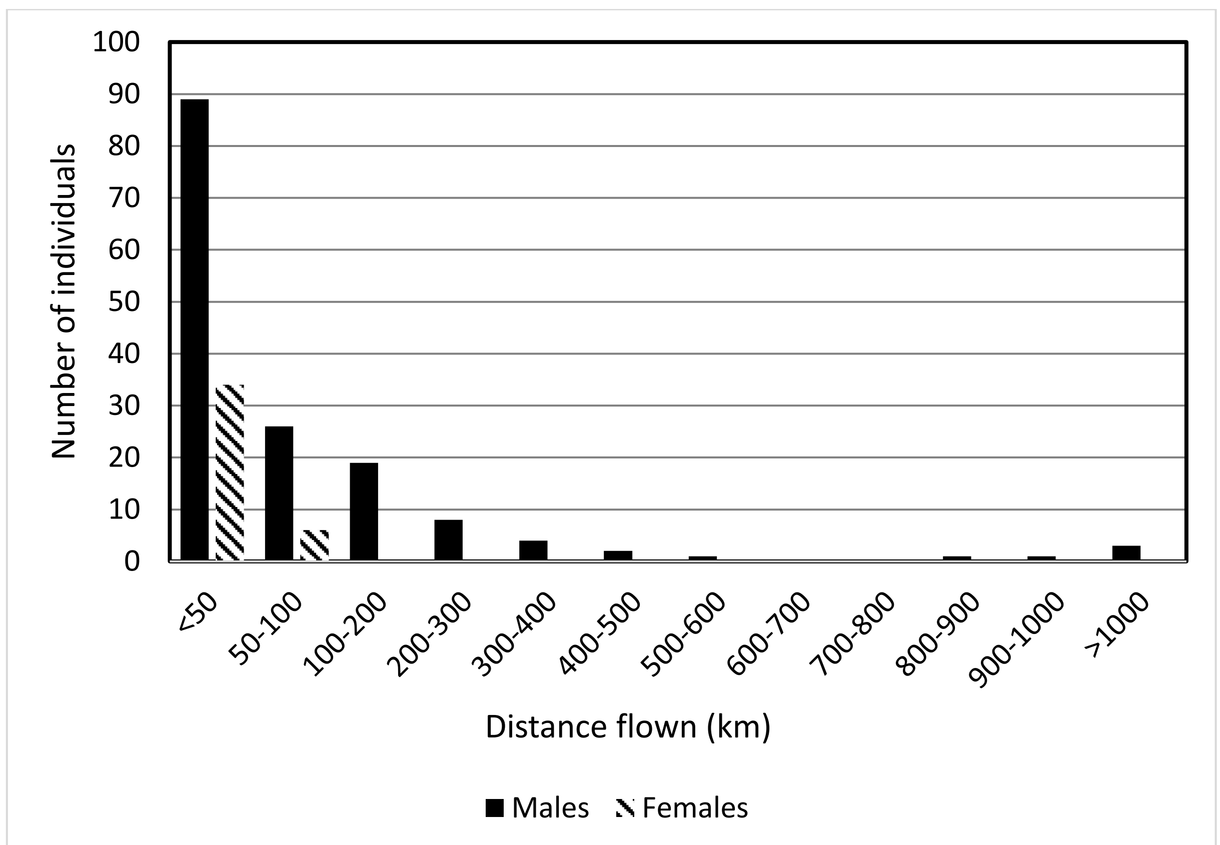

Figure 3.6 Flight distance moved by males and females of *Erebia aethiops* based on mark-release-recapture study at Smardale Gill Nature Reserve.

4. Discussion

4.1 British population genetics

The present study found that there is not clear evidence of reduced genetic diversity in populations of *Erebia aethiops* across Britain. Mean He was 0.190 (± 0.007), which is comparable with others of the *Erebia* genus. A mean He of 0.156 (± 0.029) was found for the mountain species *Erebia euryale* (Schmitt & Haubrich 2008) and a mean of 0.154 ± 0.024 for populations of *Erebia epiphron* in Pyrenees and Alps (Schmitt et al. 2006). However, levels of genetic diversity in the present study were slightly higher than those observed for the other lowland species *Erebia medusa* in the continent (mean He: 0.151 ± 0.004, Schmitt & Muller 2007) and for the Czech populations of the subspecies *Erebia epiphron silensiana* (mean He: 0.098 ± 2.6, Scmitt et al. 2005).a

There were also not significant differences among regions within Britain, consistent with previous studies by Harper (2011), who found not significant differences between core and south margin population of *Erebia aethiops* in mainland Scotland (mean He: 0.200 ± 0.005 and 0.187 ± 0.012, for core and margin populations respectively).

For a wider understanding of British *E. aethiops* diversity, the results can be compared to previous studies on other species to give an idea of the general range. AFLP analysis of non-inbred Italian goats (*Capra hircus*) showed an HE range of 0.21 – 0.24 (Ajmone-Marsan et al. 2001) and 0.10 – 0.19 in semi-isolated Herring Gull (*Larus argentatus*) populations (de Knijff et al. 2001). He can also be compared despite methodological differences so comparison is not limited to AFLP studies. Microsatellite analysis in toads showed a He range of 0.189 – 0.336 (Rowe et al. 1999). *E aethiops* He falls within these ranges, suggesting that it is comparable to other species in terms of genetic diversity.

A species known to sometimes form an inbreeding structure, the Pacific oyster (*Crassostrea gigas*) showed widely ranging He levels ranging from 0 – 0.415 and demonstrated that lower He resulted in lower fitness (Fujio 1982). In comparison, *E. aethiops* has a much narrower range and shows similar results throughout the country.

The results suggest that smaller, more isolated populations are not necessarily at risk of inbreeding. In fact, the English region showed the highest He, despite the dramatic decline in distribution over the last century (Fox et al. 2007; Fox et al. 2015). This adds to the uncertainty that small butterfly populations are automatically susceptible to extinction due to inbreeding (Schmitt et al. 2005; Harper 2011) and suggests that local extinctions may be the result of other factors, such as climate change and habitat degradation. For example, Arnside Knott showed relatively high diversity but has still

experienced a dramatic decline in recent years, with numbers recoded as part of the UK Butterfly Monitoring Scheme (UKBMS) of over 1000 individuals ten years ago to less than 200 individuals in the last 5 years.

Inbreeding can lead to reduced fitness in butterflies (Saccheri et al. 1996; Saccheri et al. 1998; Keller & Waller 2002), but this study's results suggest that *E. aethiops* is currently unaffected by diversity loss. However, the results only cover a single generation and diversity reduction could potentially occur in the smallest populations, such as Bastow Wood and Arnside Knott, in future generations (Porter & Ellis 2011).

There is also the possibility that the high levels of diversity observed in the English populations is a cause rather than an effect. It is currently uncertain why any *E. aethiops* populations survived the retraction of the southern range, but their survival following isolation could be due to a high level of initial genetic fitness; the populations may have originally been part of a much larger continuous population and survived because of their high diversity, leaving only diverse populations to include in analysis. Meanwhile, the Scottish populations may be smaller but closer together, allowing the rescue of deserted habitats by a small number of founders. However, this would require analysis and comparison of historic samples to confirm.

Differences in sample size should also be considered. Higher diversity has previously been predicted in larger sample sizes (Petit et al. 1998) when using observed heterozygosity, in this case the % of polymorphic loci, as a measure. However, an unbiased method such as expected heterozygosity (He), which assesses allelic distribution as well as frequency, has been found to be more accurate measure when dealing with uneven sample sizes (Yan & Zhang 2004; Pruett & Winker 2008). The inconsistency of the measures can be seen in Table 3.1, with Smardale Gill, the site with the largest sample size, showing the highest proportion of polymorphic loci but not the highest He of all the study populations. For this reason, polymorphic loci % cannot be considered a reliable measure of genetic diversity and only He should be accepted.

Any future study using these results should bear the uneven sample size in mind and build only on the expected heterozygosity values. Additionally, management recommendations arising from these results should not be influenced by the observed polymorphism without further analysis compensating for the uneven samples size, such as repeated random sampling of the Smardale Gill individuals using 5 samples per permutation to check the accuracy of the final measure.

Historic separation can be inferred by the degree of genetic separation between the sampled populations (Tables 3.2). The more genetically distant and differentiated populations are, the longer they have been unconnected.

Island populations have the highest degree of separation, with each island showing some connection to the Scottish mainland, but not to each other, probably due to the

length of time they have been separated. Skye shows the closest relationship to the mainland (Figure 3.2), which may be due to a more recent separation or may also be a result of butterflies crossing via the linking Skye Bridge. Alternatively, they may be crossing the water as the shortest distance between the mainland and Skye is 780m, well within the observed flight capabilities of males (Figure 3.6). If the small island of Eilean Bàn is being used as a stepping stone, the minimum required flight distance is reduced to 335m.

Relatedness values (Table 3.5) and pairwise Fst (Table 3.2) leave little doubt that Bastow Wood was repopulated with individuals from Smardale Gill. As well as showing a high level of relatedness, the Fst value of 0.005 is comparable to the results seen between connected patches in the Smardale Gill metapopulation. With a separating distance of approximately 50km, well beyond the species' flight capabilities, there is no possibility of any natural gene exchange between the two sites.

The Fst pattern may be explained by dispersal capabilities as the more geographically separate populations tend towards higher Fst (lower mixing). Additionally, postglacial colonisation may explain some of the separation as differences in isolation time and connectivity re-opening is known to influence dispersal and diversity (Hewitt 1999).

Differentiation was seen at both regional and population level. This could be due to post-glacial recolonization (Hewitt 2000) as well as human encroachment and changes in land use and retraction due to climate change (Hampe & Petit 2005). The conservation of genetically distinct populations is a particular area of concern in an era of rapid biodiversity loss (MEA 2005) and, although the species is not a current conservation priority in the UK (Fox et al. 2015), lack of action could see the loss of small, genetically unique populations.

From a regional perspective, it is possible that the differentiation contributed to the English *E. aethiops*' survival. If the populations are uniquely warm-adapted it may have allowed them to persist despite the warming effect which has driven most of the species north (Franco et al. 2006). If this is the case, the potential sources for southern reintroductions are dramatically reduced with only the English populations being suitable. However, the possibility of a warm-adapted English *E. aethiops* is purely speculative and would require further study.

Population-level differentiation means that supplementing a struggling population may be problematic as the individuals may not be able to mix. In fact, some evidence of subspecies-level differentiation in the island populations has been previously observed (Gunson 2016). As genetic distance increases with geographic distance (Figure 3.3), closer populations would be more compatible if supplementation is to be attempted.

Differentiation has also been recorded between border and margin populations of Scottish *E. aethiops* by Harper (2011). The study suggested that continued retractions could lead to the loss of genetically distinct populations unless action is taken to conserve them.

It should be mentioned that including Bastow Wood in the analysis may have reduced the overall observed level of differentiation. Bastow Wood was repopulated by an undocumented reintroduction in the 70s or 80s after the natural population went extinct in 1955 (Thomas & Lewington 2011). The original colony was morphologically distinct, so may have also been genetically distinct, whereas the reintroduced population is genetically similar to Smardale Gill (Table 3.4), highlighting one of the problems caused by unauthorised translocations.

4.2. Metapopulation genetics

Smardale Gill shows the high level of genetic diversity expected in such a large population. Saccheri et al (1998) and Hanski (2011) found that isolated populations of *Melitaea cinxia* which suffered from inbreeding (determined by % polymorphic loci) were more prone to extinction. However, in both studies, the effect was most pronounced in small populations, suggesting that the size of the Smardale population may be compensating for any negative impacts of long-term isolation.

Vandewoestijne et al. (2008) looked at expected heterozygosity in a fragmented, but well-connected *Polyommatus coridon* metapopulation in southern Belgium and found a mean He of 0.321 (± 0.056), which is slightly higher than Smardale's He (mean = 0.184, S.E. = 0.007), but not by a high amount.

The highest level of diversity seen in the largest patch, Patch 3, around the disused viaduct (Table 3.5). This suggests that there is no inbreeding depression and that the population has not experienced an historic bottleneck. Combined with the large numbers observed (Table 3.7), this indicates that Smardale Gill could be a potential source of individuals for future translocations.

The individual patches are slightly differentiated but well below the level seen in separated populations (Tables 3.3, 3.6), indicating that Smardale is a true metapopulation with connectivity between all patches. As well as genetic indications of free dispersal, mark-recapture results show that all patches are within the maximum observed male flight distance (1.5km) of at least one other patch. Additionally, there was no correlation between genetic and geographic distance, indicating that distance between patches is not a factor which significantly influences gene flow.

A total of 13 inter-patch movements were recorded from 193 recaptured individuals (6.7%). If we assume that these are representative of the whole population and 6.7% of individuals will emigrate each generation, then we can estimate that there were approximately 527 inter-patch movements from the 7869 estimated resident individuals (Table 3.7).

All inter-patch movements were performed by males. This is supported by Slamova et al (2013), who found that males are more likely to leave their natal patch. However, they also found that females are capable of longer flights (2.1km) than males (1.9km),

which is the opposite of this study's findings. Slamova et al (2006) study looked at Czech Republic *E. aethiops* populations, which suggests that there may be some behavioural and, potentially, genetic differentiation between countries as well as regions. Genetic analysis which includes other European populations would be beneficial to understand the global structure of the species.

It should be considered that the male-bias in capture and recapture rates may influence the results. Males are known to emerge earlier than females and are also more mobile due to patrolling behaviour. For this reason, it is possible that long-distance female movement did occur during the study period but was not recorded.

It was expected, due to the disproportionally large number of individuals recorded at Patch 3 (although bias may be present due to patch size), that the Smardale Gill metapopulation would function as a mainland-island metapopulation (Hanski & Simberloff, 1997), In which Patch 3 would act as a source of individuals for the surrounding smaller patches, which would be acting as island/sink populations. However, this was not the case and Patch 3 showed the highest Fst of all patches, although its Fst values are still very low (Table 3.6). Moreover, individuals were recorded moving from and to Patch 3 during the mark-release-recapture study, indicating that this patch acts both as a source and receiver of individuals from the other patches. A possible reason is that male butterflies are less likely to leave a patch with high female density (Baguette et al. 1998), so emigration may be lower overall. Another possibility is that Patch 3 was isolated for a longer period than the surrounding patches, leading to a lower level of historic mixing. However, this is unlikely given the central position occupied by Patch 3 in the metapopulation (Figure 3.5) and the very short distances to the surrounding patches, suggesting it represented the centre of the historical continuous population. Moreover, the population in Patch 8, the most isolated from the others, was only discovered in 2015 (Tom Dunbar *personal communication*), despite being on the roadside verge of a well transited road. It is likely that this population is a relatively recent colonisation from individuals potentially coming from Patch 4 (Fst=0.005), although Patch 7 is the closed in distance, it is slightly more differentiate (Fst=0.029).

Overall, the results show that *E. aethiops* are moving freely throughout the metapopulation and that connectivity is sufficient for dispersal. Even Patch 8, which is a roadside verge outside the reserve shows genetic connection to the other patches and should therefore be considered part of the Smardale Gill metapopulation for future management and conservation efforts.

For a more in-depth analysis of individual movement between patches, microsatellite analysis could be beneficial as the technique looks at far fewer loci, but is more sensitive, making it useful for studying individuals rather than entire populations (Varshney et al. 2005; Selkoe & Toonen 2006). Microsatellites are widely used in heredity studies as parentage or ancestry can be inferred from the results (Dakin & Avise 2004). Linking individuals directly to ancestors and close relatives may show

exactly when an individual dispersed from one patch to another and could be compared to factors such as yearly climate and disturbances.

4.3. Recommendations for future management

The British population of Scotch argus shows a high level of genetic diversity and is not in immediate danger from inbreeding. Therefore, conservation efforts should focus on other reasons for the species' decline in some areas, such as habitat degradation and climate change. However, inbreeding should not be ruled out as a future threat for the species if populations continue to shrink and remain small for many generations.

The potential loss of genetically differentiated populations should be a point of concern, particularly in England where they may have become warm-adapted. The loss of individuals with the ability to withstand the rising global temperature would permanently remove the possibility of any southern reintroduction.

Translocation of individuals to supplement the population at Arnside Knott is not recommended. The addition of new genetic material would only be helpful if the resident population showed signs of inbreeding, which is not the case. The cause of the species' decline at that site is currently unknown and should be determined before more butterflies are released there. If translocation were to go ahead in future, a geographically and genetically close source would be beneficial. Additional diversity is not needed, so more closely related individuals could be used to increase the likelihood of successful breeding. Smardale Gill would be the obvious choice due to its large numbers, but Crosby Garrett may be suitable depending on a population estimate.

The reintroduction of *E. aethiops* across parts of its former range in England is a possibility. Site suitability would depend on habitat quality, connectivity and management. To have the greatest chance of success, relocated individuals should be able to withstand the warmer English temperatures. Until it is determined otherwise, English individuals should be assumed to have a higher tolerance for heat and should be used as a source. Again, Smardale Gill would be the most suitable site.

If Smardale Gill is used as a source for future translocations or reintroductions, a single patch need not be targeted. As all patches show free mixing, individuals could be taken from any part of the reserve depending on numbers and ease of capture. If adults are removed, doing so prior to peak flight time would be advisable, to allow females to successfully mate and lay in the new location.

For any *E. aethiops* reintroduction, the lazy behaviour of females should be taken into account. Results show that they do not move from their natal patch, with a maximum flight distance of 80m (Figure 3.6). Unless patches are very close together, the creation of a fully connected metapopulation will require females to be released in every patch. Males are more mobile and will disperse naturally, but female sedentariness should be a focal point of any reintroduction planning.

The population at Smardale Gill is faring well and current management practices appear sufficient. Crosby Garrett is genetically robust, but a population estimate would be useful to assess the state of the colony and its suitability as a potential source in future. Bastow Wood would also benefit from a population count and additional monitoring for inbreeding in future as a founder effect may still become apparent. Arnside Knott is genetically healthy, but the numbers are dropping dramatically. This site should be a conservation priority as its loss would also mean the loss of a genetically distinct population. Further genetic analysis in the future would be useful to determine whether a bottleneck is currently occurring at this population and the effect it may have in its long-term persistence.

As the extraction of DNA from a single leg proved successful, it is recommended that future genetic studies employ this method to avoid removing individuals from the population. This is particularly important when sampling small populations such as Arnside Knott, or when sampling females.

A generalised conclusion would be that small, fragmented populations do not always lead to inbreeding and that other factors may be responsible for decline. Additionally, a genetically robust population is not necessarily safer than one with lower diversity, as demonstrated by the Arnside Knott decline.

Reintroduction and translocation should not only be considered when an entire species is at risk, but when separate populations with potentially beneficial adaptations are threatened. As climate change continues to drive the species north, the best hope for its future survival may lay with the surviving southern populations. However, as many reintroductions fail, understanding the species abilities and requirements is essential. It may be more practicable to use management to mitigate the negative impacts at the current location, especially when a population is small and diverse, so is unlikely to suffer from inbreeding as it grows.

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Appendices

Appendix 1. DNA sample table

Sample #	Site	Date Collected	Leg/Whole	Sex	Successfully Analyzed?
A1	Insh Marshes	26/08/2015	Whole	Male	Yes
A2	Insh Marshes	26/08/2015	Whole	Male	Yes
A3	Insh Marshes	26/08/2015	Whole	Male	Yes
A4	Insh Marshes	26/08/2015	Whole	Male	Yes
A5	Insh Marshes	26/08/2015	Whole	Male	Yes
B1	Craigower	26/08/2015	Whole	Male	Yes
B2	Craigower	26/08/2015	Whole	Male	Yes
B3	Craigower	26/08/2015	Whole	Male	Yes
B4	Craigower	26/08/2015	Whole	Male	Yes
B5	Craigower	26/08/2015	Whole	Male	Yes
C1	Tomnavoulin	27/08/2015	Whole	Male	Yes
C2	Tomnavoulin	27/08/2015	Whole	Male	Yes
C3	Tomnavoulin	27/08/2015	Whole	Male	No
C4	Tomnavoulin	27/08/2015	Whole	Male	Yes
C5	Tomnavoulin	27/08/2015	Whole	Male	Yes
D1	Skye	20/08/2015	Whole	Male	Yes
D2	Skye	20/08/2015	Whole	Male	Yes
D3	Skye	20/08/2015	Whole	Male	Yes
D4	Skye	20/08/2015	Whole	Male	Yes
D5	Skye	20/08/2015	Whole	Male	Yes
E1	Mull	22/08/2015	Whole	Male	No
E2	Mull	22/08/2015	Whole	Male	Yes
E3	Mull	22/08/2015	Whole	Male	Yes
E4	Mull	22/08/2015	Whole	Male	Yes
E5	Mull	22/08/2015	Whole	Male	Yes
F1	Arran	23/08/2015	Whole	Male	Yes
F2	Arran	23/08/2015	Whole	Male	Yes
F3	Arran	23/08/2015	Whole	Male	Yes
F4	Arran	23/08/2015	Whole	Male	Yes
F5	Arran	23/08/2015	Whole	Male	Yes
CG1	Crosby Garrett	22/08/2017	Whole	Male	Yes
CG2	Crosby Garrett	22/08/2017	Leg	Female	Yes

CG3	Crosby Garrett	22/08/2017	Leg	Female	No
CG4	Crosby Garrett	22/08/2017	Leg	Female	Yes
CG5	Crosby Garrett	22/08/2017	Leg	Female	Yes
AK1	Arnside Knott	20/08/2017	Whole	Male	Yes
AK2	Arnside Knott	20/08/2017	Whole	Male	Yes
AK3	Arnside Knott	24/08/2017	Leg	Female	Yes
AK4	Arnside Knott	24/08/2017	Leg	Female	Yes
AK5	Arnside Knott	24/08/2017	Leg	Female	Yes
AK6	Arnside Knott	24/08/2017	Leg	Female	Yes
BW1	Bastow Wood	25/08/2017	Whole	Male	Yes
BW2	Bastow Wood	25/08/2017	Whole	Male	Yes
BW3	Bastow Wood	25/08/2017	Whole	Male	Yes
BW4	Bastow Wood	25/08/2017	Leg	Female	Yes
BW5	Bastow Wood	25/08/2017	Leg	Female	Yes
BW6	Bastow Wood	25/08/2017	Leg	Female	Yes
BW7	Bastow Wood	25/08/2017	Leg	Female	Yes
MR1	Smardale Gill	22/08/2017	Whole	Male	Yes
MR2	Smardale Gill	22/08/2017	Whole	Male	Yes
MR3	Smardale Gill	23/08/2017	Whole	Male	Yes
MR4	Smardale Gill	27/08/2017	Leg	Female	Yes
MR5	Smardale Gill	23/08/2017	Leg	Female	Yes
MR6	Smardale Gill	01/09/2017	Leg	Female	Yes
RS1	Smardale Gill	22/08/2017	Whole	Male	Yes
RS2	Smardale Gill	22/08/2017	Whole	Male	Yes
RS3	Smardale Gill	22/08/2017	Whole	Male	Yes
RS4	Smardale Gill	22/08/2017	Whole	Male	Yes
RS5	Smardale Gill	22/08/2017	Whole	Male	Yes
BS1	Smardale Gill	21/08/2017	Whole	Male	Yes
BS2	Smardale Gill	21/08/2017	Whole	Male	Yes
BS3	Smardale Gill	21/08/2017	Whole	Male	Yes
BS4	Smardale Gill	21/08/2017	Whole	Male	Yes
BS5	Smardale Gill	21/08/2017	Whole	Male	Yes
BS6	Smardale Gill	21/08/2017	Whole	Male	Yes
AP1	Smardale Gill	01/09/2017	Whole	Male	Yes
AP2	Smardale Gill	01/09/2017	Whole	Male	Yes
AP3	Smardale Gill	01/09/2017	Whole	Male	Yes
AP4	Smardale Gill	01/09/2017	Whole	Male	Yes
AP5	Smardale Gill	30/08/2017	Whole	Male	Yes
HK1	Smardale Gill	27/08/2017	Whole	Male	Yes
HK2	Smardale Gill	27/08/2017	Whole	Male	Yes

HK3	Smardale Gill	27/08/2017	Whole	Male	Yes
HK4	Smardale Gill	22/08/2017	Whole	Male	Yes
HK5	Smardale Gill	22/08/2017	Whole	Male	Yes
V1	Smardale Gill	22/08/2017	Whole	Male	Yes
V2	Smardale Gill	22/08/2017	Whole	Male	Yes
V3	Smardale Gill	21/08/2017	Whole	Male	Yes
V4	Smardale Gill	21/08/2017	Whole	Male	Yes
V5	Smardale Gill	21/08/2017	Whole	Male	Yes
V6	Smardale Gill	21/08/2017	Whole	Male	Yes
V7	Smardale Gill	21/08/2017	Whole	Male	Yes
V8	Smardale Gill	21/08/2017	Whole	Male	Yes
V9	Smardale Gill	23/08/2017	Whole	Male	Yes
V10	Smardale Gill	07/08/2017	Whole	Male	Yes
V11	Smardale Gill	09/08/2017	Whole	Male	Yes
V12	Smardale Gill	13/08/2017	Whole	Male	Yes
V13	Smardale Gill	09/08/2017	Whole	Male	Yes
V14	Smardale Gill	23/08/2017	Whole	Male	Yes
V15	Smardale Gill	21/08/2017	Whole	Male	Yes

Appendix 2. DNA extraction base protocol from DNeasy 96 Blood and Tissue kit

The DNeasy 96 Blood & Tissue Kit (cat. nos. 69581 and 69582) can be stored at room temperature (15–25°C) for up to 1 year if not otherwise stated on label.

Further information

- DNeasy Blood & Tissue Handbook: www.qiagen.com/HB-2061
- Safety Data Sheets: www.qiagen.com/safety
- Technical assistance: support.qiagen.com

Notes before starting

- Perform all centrifugation steps at room temperature (15–25°C).
- If necessary, redissolve any precipitates in Buffer AL and Buffer ATL.
- Add ethanol to Buffer AW1 and Buffer AW2 concentrates.
- If using tissue, add ethanol to Buffer AL before use.
- If using animal blood, refer to the handbook.
- Equilibrate frozen tissue samples to room temperature.

 Preheat an incubator to 56°C.

1. Cut ≤20 mg tissue into small pieces, and place into a collection microtube. For rodent

tails, use 1 (rat) or 2 (mouse) 0.4–0.6 cm lengths of tail. Use a 96-Well-Plate Register for

sample position.

2. Prepare a working solution containing 20 µl proteinase K stock solution and 180 µl

Buffer ATL per sample, and mix by vortexing. Immediately pipet 200 µl working solution

into each collection microtube. Tightly seal the microtubes using the caps provided.

3. Place the clear cover over each rack, and mix by inverting. Centrifuge to collect any

solution from the caps. The samples must be completely submerged in the

proteinase K–Buffer ATL solution after centrifugation.

4. Incubate at 56°C overnight or until the samples are completely lysed. Place a weight on top

of the caps during the incubation. Mix occasionally during incubation to disperse the sample.

5. Ensure that the microtubes are properly sealed. Cover the racks and vigorously shake up

and down for 15 s. Centrifuge to collect any solution from the caps. Ensure that samples

are completely lysed to avoid clogging wells of the DNeasy 96 plate.

6. Carefully remove the caps and add 410 µl Buffer AL–ethanol mixture to each sample,

and tightly reseal using new caps.

7. Place a clear cover over each rack and shake the racks vigorously up and down for

15 s. Centrifuge to collect any solution from the caps.

8. Place 2 DNeasy 96 plates on top of S-Blocks. Mark the DNeasy 96 plates for later

sample identification.

9. Carefully remove microtube caps and transfer the lysate (maximum 900 µl) of each

sample to each well of the DNeasy 96 plates.

10.Seal each plate with an AirPore Tape Sheet. Centrifuge for 10 min at 3800 x g (6000 rpm).

11.Remove the tape. Add 500 µl Buffer AW1 to each sample.

12.Seal with a new AirPore Tape Sheet. Centrifuge for 5 min at 3800 x g.

13.Add 500 µl Buffer AW2 to each sample.

14.Centrifuge for 15 min at 3800 x g (do not seal the plate with tape).

15.Place each DNeasy 96 plate on a new rack of Elution Microtubes RS.

16.Add 200 µl Buffer AE to each sample, and seal with new AirPore Tape Sheets. Incubate

for 1 min at room temperature (15–25°C). Centrifuge for 2 min at 3800 x g.

Optional: repeat this step for increased DNA yields.

17.Seal the Elution Microtubes RS with new caps to store the eluted DNA.

Appendix 3. AFLP protocol

AFLP Reactions – MRR lab protocol

Reagents:

EcoRI	–	NEB Cat# R0101S 10,000 U @ 20 U/µl
MseI	–	NEB Cat# R0525S 500 U @ 10 U/µl
T4 DNA ligase	–	NEB Cat# M0202S
Taq DNA polymerase	–	NEB Cat# M0320L 2000 U @ 5 U/ µl
dNTP stocks	–	NEB Cat# N0447S 4 x 0.2 ml @ 10 mM each dNTP

Preparation of adapters

Prepare adapters freshly each time.

Heat the required amount of adapters to 95°C for 5 min, and allow to cool gradually to room temp.

For 5 µM EcoRI adapter:

1:1:18 ratio of 100 µM EcoRI A1: 100 µM EcoRI A2:ddH_2O

For 50 µM MseI adapter:

1:1 ratio of 100 µM - MseI A1 and 100 µM MseI A2

Restriction-Ligation step

Create a master-mix for the number of reactions required based on the volumes per reaction below:

Reagent	Volume - µl
10x T4 DNA ligase buffer	1.1
0.5 M NaCl	1.1
1 mg/ml BSA	0.55
5 µM EcoRI adapter	1.0
50 µM MseI adapter	1.0
MseI (10U/µl)	0.1
EcoRI (20U/µl)	0.25
T4 DNA ligase (NEB; 400U/µl)	0.6

Mix thoroughly and collect by a brief centrifugation.

Aliquot 5.5 µl master mix per sample into tubes and add 5.5 µl DNA sample at 10 ng/µl.

Mix and centrifuge briefly.

Incubate for 37°C for 2 hours and then 17°C overnight.

Use either a thermal cycler with heated lid, else add a drop of mineral oil on top of the reaction to prevent evaporation.

On completion, add 190 µl ddH_2O.

Pre-selective amplification

Create a master-mix for the number of PCR reactions required based on the volumes per reaction below:

Reagent	Volume - µl
ddH_2O	12.0
10x PCR buffer	2.0
25 mM $MgCl_2$	1.2

5 µM Preselective MseI primer	1.1
5 µM Preselective EcoRI A or B primer	1.1
10 mM dNTP mix	0.4
Taq DNA polymerase (NEB 5 U/µl)	0.2

Add 18 µl PCR master-mix to 2 µl restriction-ligation reaction products.

Mix and collect by a brief centrifugation.

Run in thermal cycler as follows:

One cycle of 72°C – 2 min

20 cycles of:

94°C – 20 s

56°C – 30 s

72°C – 1 min

One cycle of 60°C – 15 min

After PCR, dilute 1:20 with dH_2O

Selective amplification

Primer stocks:

Fluorescently-labelled EcoRI primers
at 1 µM

'Selective EcoRI-TCT FAM'
'Selective EcoRI-TGA HEX'
'Selective EcoRI-ATC ATTO'

Selective MseI primers at 5 µM

'Selective MseI-CAA'
'Selective MseI-CTG'

Primer pairs used at York to generate AFLP markers for *E. aethiops* :

EcoRI-TCT and MseI-CAA
EcoRI-TGA and MseI-CTG
EcoRI-ATC and MseI-CTG

Create a master-mix for each of the three primer combinations containing enough reagents for the number of pre-selective PCR samples to amplify, as follows:

Reagent	**Volume - µl**
ddH_2O	5.2
10x PCR buffer	1.0
25 mM $MgCl_2$	0.6
5 µM Selective MseI primer	0.5
1 µM Selective EcoRI primer	0.5
10 mM dNTP mix	0.2
Taq DNA polymerase (NEB 5 U/µl)	0.05

Add 8 µl PCR master-mix to 2 µl of diluted pre-selective PCR reaction products.

Mix and collect by a brief centrifugation.

Run in thermal cycler as follows:

One cycle of 94°C – 2 min

9 cycles of:
94°C – 30 s
65°C -1°C /cycle – 30 s
72°C – 2 min

23 cycles of:
94°C – 30 s
56°C – 30 s
72°C – 2 min

One cycle of 72°C – 10 min