The Conservation of Climatically Adaptive Genetic Variation

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by

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Contents

Abstract

Anthropogenic climate change poses a significant threat to plant populations and communities because the resulting environmental changes are expected to exceed the capacity of the individuals comprising many populations to reproduce or survive. Evolutionary adaptation can provide plant populations with the capacity to alter their climate tolerance, buffering them against the adverse effects of climate change, but requires the presence of climatically-adaptive standing genetic variation. Microclimatic variation—variability in climatic conditions over fine spatial scales—represents one potential source of such genetic variation, because locally prevailing climatic conditions differ from those in the surrounding areas, and have the potential to drive selection for variant genotypes that confer fitness advantages under those specific conditions, supporting diversity at larger spatial scales. Little is known about the capacity of microclimatic variation in landscapes to support climatically adaptive genetic variation and provide a buffer against future climate change. Furthermore, the success of ecological restoration projects that create new populations and habitats from seed might depend upon the inclusion of relevant climatically adaptive variation, yet we know almost nothing regarding the impacts of seed production techniques on the maintenance of these variants within seed stocks used in restoration. In this thesis I investigate the extent to which microclimatic conditions drive selection for distinct phenotypes and genotypes that may be climatically adaptive, in the ecological model forage grass *Festuca ovina*. I use two different study systems to address these issues, one based on naturally-occurring microclimatic variation across a landscape, and one in which climate conditions have been experimentally manipulated for almost 30 years. Additionally, I investigate the effects of commercial seed production on genetic diversity within *F. ovina*, with the aim of assessing the maintenance of climatically adaptive genetic variation in ecological restoration projects. I found that microclimatic variation linked to north- and south-facing valley slopes in the Peak District (UK) is consistently associated with a suite of plant phenotypes and genotypes that are likely to be adaptive in the distinct conditions of different microclimates. *F. ovina* populations from cooler/ wetter microclimates tend to prioritise vegetative growth over reproduction, potentially increasing their ability to compete in these environments, whereas those from warmer drier microclimates prioritise sexual reproduction, which may increase the capacity of

populations to persist through stressful abiotic conditions. Gene loci that are carry a signature of climatically adaptive genetic differentiation had functions linked to stress tolerance, reproductive timing and disease resistance. I documented similar genomic differentiation between experimental climate treatments at the Buxton Climate Change Impacts Laboratory, and furthermore provide evidence that the evolutionary changes there are functionally equivalent to those seen in naturally occurring microclimate populations in the wider Peak District. Finally, I compared the genetic structure of a wild *F. ovina* source population to later generation artificial populations derived from the source for commercial seed production and ecological restoration at Emorsgate Seeds (Norfolk, UK). Here I found that there was no signal of artificial selection during the process of raising seed commercially, between the wild source population and the fourth and sixth generation of cultivated stock. The results presented here demonstrate that microclimatic heterogeneity provides an important source of naturally occurring, climatically-adaptive genetic variation, with the potential to buffer populations against the effects of climate change by enabling adaptive evolutionary responses. Furthermore, the facts that *F. ovina* is obligately outcrossing, long-lived and wind pollinated appears to encourage retention of genetic variation within populations, including under long term cultivation for ecological restoration. On the basis of my results, I recommend that microclimatic heterogeneity and associated climatically adaptive variation should be carefully considered in conservation projects and in projections of species' responses to climate change. Furthermore, the existence of climatically relevant variation in plant phenotypes and genotypes suggests that seed sourcing and commercial propagation should be undertaken to capture and retain as much of this variation as possible.

1 General introduction

1.1 Climate, microclimate and climate change

1.1.1 Climate and microclimate

The lives of plants are intrinsically linked to climate; climate refers to long term meteorological averages for climatic variables – such as precipitation and air temperature – for an area (Maslin, 2013). The climatic variables of greatest importance to plants include temperature, humidity, precipitation and wind. Plant species' geographical distribution and the fitness of individual plants is strongly dictated by the annual averages, extremes and patterning of these variables at both temporal and spatial scales (Austin & Van Niel, 2011; Banerjee *et al.*, 2019).

Climatic variation affecting plant growth and survival can be observed at different spatial scales categorised broadly into the "macroclimate" and the "microclimate". Macroclimate describes the conditions across larger geographical areas or meteorological zones. Microclimates are climatic conditions within much more localised geographical spaces caused by physical features of the landscape. Features including topography, watercourses, tree cover and even individual rocks have a controlling influence on conditions. Microclimate sites mostly differ in water availability and average temperature from the surrounding environment, caused by protection from or increased exposure to the drying and thermal effects of insolation and wind (Stoutjesdijk & Barkman, 2015).

Topographical slope aspect—the geographical orientation of a hillside, valley or piece of land— is a major source of microclimatic variation (Copeland & Harrison, 2015; Giaccone *et al.*, 2019). In the northern hemisphere, north facing slopes receive both a lower total exposure time to insolation and lower energy transfer per unit area during insolation than south facing slopes. The latter effect arises because the sun strikes north facing slopes at a more acute angle. Geographically proximate north and south-facing slope aspects can differ significantly from each other in their microclimate by several degrees in air and soil-surface temperatures and in soil moisture. As a resultant of these climatic differences, adjacent slopes can differ significantly in plant community composition (Giaccone *et al.*, 2019; Rorison *et al.*, 1986).

1.1.2 Temporal aspects and climate change

Seasonality refers to the temporal patterning of climatic variation within a year, for example whether precipitation is distributed evenly or concentrated primarily within 6 months. Plant survival is dependent not just on the annual averages but on the variation and interaction of these variables throughout the year. In temperate regions it matters whether sufficient rain falls not just over the whole year, but during the times where temperature permits active plant growth (Banerjee *et al.*, 2019). The seasonal variation of a location is of vital importance to understanding the ecology of plants there.

The last two centuries of human activity have significantly affected global climate, far greater than previous rates of change. Greenhouse gas emissions from burning fossil fuels over the last 150 years have massively accelerated the rate of change, having increased global temperature by 1°C already; global average temperature is predicted to rise 4°C in the next century without intervention (IPCC, 2014). Climate change is already causing problems for plant conservation as species struggle to adapt fast enough to the new conditions, an issue which is only predicted to worsen without mitigation in coming years.

1.2 Impacts of the climate on plant geographical ranges and population biology

1.2.1 Distribution and niche

Plant species' ranges, their physical distribution in space, are linked to climatic conditions. Species have upper and lower temperature bounds outwith which they cannot survive, excluding them from all locations regularly passing these thresholds. Water availability below a plant's requirement for survival, annually or during critical growth periods, will similarly shape species' geographical distributions. The sum of the environmental requirements tolerable for survival of a species make up the species' niche (Begon *et al.*, 2006). These climatic conditions define the *potential* range of a plant species, i.e. all the places it can potentially survive (Brown, 1984; Holt, 2003; Martin & Husband, 2009).

The realised range of a species described the locations where it is actually present. The realised range of a species is rarely equivalent to its potential due to factors such as competitive exclusion, dispersal barriers and historical climatic conditions (Brown, 1984;

Holt, 2003; Martin & Husband, 2009; Sporbert *et al.*, 2020). Competitive exclusion and niche partitioning interact with microclimate, altering which species or genotype prevails in a particular location (Bohner & Diez, 2020; Bullock *et al.*, 2000). Many of the biotic and abiotic factors controlling plant distribution are strongly influenced by climate.

Microclimate influences the suitability of a site for a particular species relative to the surrounding area. Hills and valleys create large patches of microclimatically differentiated habitat controlled by slope aspect; the angle of incidence of the sun and total daily insolation affect air and soil temperature, altering rates of water loss, plant growth and susceptibility to drought (Stoutjesdijk & Barkman, 2015). The consistency of these difference and the size of the topographical features that create them mean slope aspect microclimates can have important roles in shaping plant distribution.

1.2.2 Populations, metapopulations and demographics

Populations may be defined as "*A group of conspecific individuals that is demographically, genetically, or spatially disjunct from other groups of individuals*" (Wells & Richmond, 1973). It is often useful to subdivide and group populations into subpopulations and metapopulations. A subpopulation is a, usually spatially, defined subset of individuals that makes up part of a larger population. Populations and subpopulations are not arbitrary but exist as part of a nested hierarchy of grouped individuals.

A metapopulation is a collection of populations that are spatially disparate but are linked by a low level of migration and mating. In plants this is usually through dispersal of propagules or pollen transfer between patches (Wells & Richmond, 1973). Populations growing under unfavourable climatic conditions for seed set may be reliant on immigration from more fecund adjacent populations, a source-sink dynamic. Such populations risk extirpation if changing climate conditions move further from the optimum or negatively impact their source population (Crawley, 1990).

Plant species can have exceptionally long reproductive lifespans, with demographic turnover mediated by environmental disturbance (Coffin & Urban, 1993; Crawley, 1990; De Witte & Stöcklin, 2010). Theoretically a plant population on a consistently damp slope may have very different rates or recruitment from seed than one on a slope subject to regular extreme drought. Different environmental stressors may select for either high investment in

vegetative or sexual reproduction, with populations locally adapted to these conditions (Ravenscroft *et al.*, 2014).

1.2.3 Dispersal and range shift

The distribution of plant populations in space is influenced not just by local climate, but by the climate in adjacent areas. A stretch of climatically inhospitable habitat greater than the maximum dispersal distance between suitable patches constitutes a physical barrier to colonisation (Lomolino & Lomolino, 2020). Zoochorous plants' distribution may reflect the climatic limitations/preferences of their dispersal partners rather than the plants themselves (Mackay & Gross, 2019; Sales *et al.*, 2020). The absence of species from environmentally suitable habitat patches may sometimes be explained by climate mediated dispersal barriers.

Changing climate shifts the potential range of plant species. As an extreme example: during the Last Glacial Maximum all vascular plants were excluded from much of northern Eurasia by an ice-sheet (Evans & Evans, 2018). With the rapid retreat of the ice-sheets at the end of the Younger Dryas (≈11.7kya), vascular plants recolonised from refugia in southern Eurasia (Hodková *et al.*, 2019; Juřičková *et al.*, 2018; Kinloch *et al.*, 1986). This shift in range is known to significantly lag behind the change in climate (Butterfield *et al.*, 2019). What is unknown is how anthropogenic climate change will affect plant populations and movement in response to the current, rapid rate of change, and what mechanisms may be important in mitigating the negative effects of climate change.

1.3 Evolution, local adaptation and genetic drift

All organisms are evolved to suit the selective pressures of their environments, including climate. Plant adaptive responses to climate consist of both fixed and plastic traits (Franks *et al.*, 2014; Hoffmann & Sgrò, 2011; Jump & Peñuelas, 2005). Short term change and unstable climatic conditions favour reliance on trait plasticity, allowing individuals to alter their physiology as needed, within a set range. Longer term and more stable changes favour the evolution of fixed traits specific to the environment. Most plant adaptive traits show a mixture of plasticity and specificity to a climate optimum for a population (Power *et al.*, 2019; Ren *et al.*, 2020).

A locally adapted population is one in which the relative fitness is higher *in situ* than that of a genotype introduced from another location (Kawecki & Ebert, 2004). Climate is a major selection pressure driving local adaptation (Fridley *et al.*, 2007). Plants, as sessile organisms often with large effective populations, show strong selection for locally adaptive traits.

The phenology of European Scots pine (*Pinus sylvestris*) provides a good example of local adaptation to climate (Hurme *et al.*, 1997). Growing from the northern treeline to mountains in the Mediterranean, northern populations are adapted to longer winter/shorter summers and a sharper seasonal transition than southern populations. With a shorter growing season and the need for earlier bud set and frost hardening, in a common garden northern genotypes cease annual growth earlier than southern. This fixed difference in the environmental thresholds triggering winter quiescence means that, compared to southern accessions, northern accessions grow slower at southern latitudes, but show greater survivorship on home turf.

Variation in local climatic conditions can lead to genetic differentiation between populations at adaptively neutral loci. Genetic drift is a neutral evolutionary change that can be observed between populations and within populations over time (Masel, 2011). These changes in the relative frequency of selectively neutral alleles can occur as a stochastic effect of maintaining two separate genepools. Genetic differences between populations can be the result of drift rather than a marker of different selective pressures.

Genetic drift can also be a result of population bottlenecks – events in which the effective population size is dramatically reduced (Masel, 2011). Causes of genetic bottlenecks may include founder effects in the colonisation of newly available habitat, disturbance events, strong selective sweeps and range contraction. The linkage between adaptively neutral genes and genes under selection can make neutral loci appear to be under selection, rather than an incidental effect of the selection. When attempting to identify not just signals of selection but specific genes or loci under selection it is important to keep this in mind.

1.4 Reviewing evidence for climate as a driver of genetic change

1.4.1 Genome-climate association studies

Studies that seek to associate genotypes with climatic factors are widely used e.g. (Günther *et al.*, 2016; Yang *et al.*, 2016; Zhang *et al.*, 2014). Yang *et al.* used AFLP to examine 11 populations of *Liriodendron chinense*, a magnoliid tree native to central and southern China. They analysed 276 individuals for outlier loci, identifying 6 of the 9 outliers as likely associated with local climate adaptation. The other three outlier loci were strongly differentiated between populations but not in association with climatic factors, likely representing differentiation due to population history or to other selection pressures. These studies excel at capturing data from large numbers of individuals and populations, providing strong evidence for genetic differentiation between populations.

Günther *et al.*'s paper demonstrates one of the vital assumptions of these studies: Consistent genetic differences between populations consist of a mixture of adaptive genes and neutral loci. Their study compared high and low altitude populations of *A. thaliana* on different peaks in the Italian alps. Two high altitude populations, 40km apart and separated by the Adige river valley, were discovered to be more closely related to each other than they were to their nearest low altitude counterparts. The high and low altitude populations are thought to represent multiple waves of post glacial recolonization from refugia with different upper altitude limits. This leads to the conclusion that while some of the genes strongly differentiated between upper and lower populations are adaptive, others are due to genetic drift between the refugial source populations.

The molecular methods used in genome-climate association influence the specificity of the resultant data. In the *L. chinense* paper data was from AFLP which tells you that there are regions that are differentiated and/or under selection. Others such as Zhang *et al.*'s single gene investigation in *Hordeum sponteneum* and Günther *et al.*'s whole genome population work on *A. thaliana* generate sequence data that can be mapped to specific genes and suggest interrelationships between alleles and environmental factors.

1.4.2 Common gardens

Common gardens consist of genotypes from different populations grown together to observe trait differences under controlled environmental conditions (Aspinwall *et al.*, 2013; Preite *et al.*, 2015; Richardson *et al.*, 2017; Ward, 1969). Common gardens show innate trait differences between populations, which cannot necessarily be measured *in situ*. Differences in phenology and morphology between populations from different latitudes or microclimates can be a plastic response to *in-situ* conditions. Plants are well known for their plasticity, thus, phenotypic differences observed in the common garden can be reliably confirmed as being genetic in origin and more indicative of local adaptation.

Aspinwall *et al.* conducted a common garden experiment with the C_4 grass *Panicum virgatum* using nine genotypes from wild and semi-wild populations from varying temperature and precipitation origins across the United States. They found flowering time, productivity, leaf and tiller traits and leaf nitrogen content under common garden conditions correlated strongly with mean annual temperature of origin. Their findings suggest either adaptive significance for temperature or to downstream/correlated effects[1]. Morphological traits like these, associated with particular environmental stressors, can be used to infer the nature of local adaptation. However, as with genetic association studies observed differences may represent demographic history rather than local adaptation, but the ability to identify trait differences not due to plastic responses to *in situ* climate can be informative.

1.4.3 Reciprocal transplants

The gold standard experiment for confirming local adaptation in plants is the reciprocal transplant experiment (Bemmels & Anderson, 2019; Macel *et al.*, 2007; Walker *et al.*, 2019). Reciprocal transplants investigate fitness by transplanting individuals between locations and directly comparing traits within and between sites. Though they do not identify specific genes involved, by definition reciprocal transplant experiments can conclusively

[1] Extreme summer highs/winter lows, yearly drought stress and thermal niche of herbivores and pathogens may all be intertwined with mean annual temperature and may provide the selective force behind the observable differences.

demonstrate local adaptation through observation of fitness effects under specific local conditions.

Macel *et al.* performed a reciprocal transplant experiment on the grass *Holcus lanatus* and the forb *Lotus corniculatus* aiming to investigate local adaptation to soil conditions and climate. They created common gardens at three sites along a coastal-continental gradient in Europe (UK, Czechia and Switzerland) where both species grow. Plants were grown at their own site in their native soil, and at both other sites in either their own soil or soil from that site. The grass displayed no observable effect from soil type, but strong fitness and growth advantages at their home sites, indicating strong local adaptation associated with climatic conditions. *L. corniculatus* however showed no observable effect from site, though a significant difference in fruit numbers related to soil type, suggesting only weak local adaptation to climatic conditions.

When investigating climate, studies must account for edaphic and biotic factors that can influence growth rates and fitness including herbivory and pathogens – factors that themselves may be controlled by climatic conditions. Even with excellent experimental design, climate consists of interacting variables (e.g. mean annual/monthly temperature, length of growing season and monthly water availability); unless researchers can find sites perfectly matched for all but one value the specific environmental factors driving selection can be hard to identify. None the less, reciprocal transplant experiments remain powerful tools for identifying and confirming local adaptation using real world conditions and natural plant populations to assess home field advantage and the relative importance of different factors in driving selection.

1.4.4 Climate manipulation studies

Combining the strengths of reciprocal transplants and common gardens are climate manipulation studies (Harter *et al.*, 2015; Matesanz *et al.*, 2020; Ramírez-Valiente *et al.*, 2017; Saeidnia *et al.*, 2020). Climate manipulation studies involve manipulating climate *in-* or *ex- situ*, often for several years (Ravenscroft *et al.*, 2015; Richardson *et al.*, 2017; Trinder *et al.*, 2020; Whitney *et al.*, 2019). The aim of these studies is to alter one or more specific climate variables while keeping all others constant, to try and identify specific factors and responses involved in plant adaptation.

Ramírez-Valiente *et al.* conducted a climate manipulated common garden study on the neotropical live oak *Quercus oleoides*, studying artificial precipitation regimes on seedlings. They used 634 plants from eight locations across Central America and four treatments manipulating wet and dry season water availability representing climatic regimes seen throughout the species' range. They were able to quantify trait differences between seedlings from different environments, alongside consistent but plastic differences between treatments based on maternal habitat water availability. The correlation between certain traits with plastic responses to water and maternal water regime provides strong evidence of adaptation to water availability.

Long term climate manipulation studies are rare. They are time consuming, expensive and require funding over time periods longer than the average grant. In Britain, the longest running in-situ climate manipulation experiment is the Buxton Climate Change Impacts Laboratory, at over twenty-five years of constant climate manipulation of a natural calcareous grassland. Ravenscroft *et al.* used AFLP investigate genetic divergence between the different treatments after fifteen years. *Festuca ovina* and *Plantago lanceolata*, a grass and a forb abundant at the site, were chosen for study. They investigated the genotypes of both species under the following treatments: warming[2], drought[3], watering[4], control plots[5] and two combination treatments of warming+drought and warming+watering. While outliers were not detectable for *F. ovina* using Bayscan, *P. lanceolata* showed two outlier loci associated with climate treatment. Pairwise comparison between control and treatments showed significant genetic differentiation between control plots and treatments for both species. This study demonstrated genetic differentiation in response to climate for both species in response to fifteen years (≤15 generations) of manipulation.

These studies obviously provide us incredibly compelling evidence for climate as a causal driver of genetic change, however, grant based research economies do not favouring multi-decade projects. They are only feasible for certain types of habitat; grasslands, short herbs and low shrub communities are easily navigable and suited to the installation of rain shelters, irrigation, heating cables etc. Overall, they are an incredibly powerful tool in

[2] soil surface temperature maintained 3°C above ambient between November and April
[3] automatic rain shelters prevent rainfall on plots in July and August
[4] plots are irrigated at 20% above average monthly rainfall between June and September
[5] no treatment applied

investigating the processes underlying climate driven genetic change, providing powerful evidence, though the scope of such projects tends to be limited by practical concerns.

1.4.5 Conclusions

The approaches discussed above have their strengths and weaknesses in identifying local adaptation to climate. Genome-climate association studies are powerful and can screen large numbers of plants and whole populations for genetic differentiation, if not always local adaptation. Common gardens are valuable in their ability to untangle the relationship between plasticity and innate differences between genotypes from differing climatic backgrounds. However, the impact of edaphic factors on plants in common gardens cannot be ignored (Macel *et al.*, 2007).

1.5 Conservation of plant biodiversity in a changing climate

1.5.1 Current threats to plants

There are few studies documenting plant species already lost to climate change, as it is hard to measure and the current increase is still apparently minor, however extinctions are predicted to increase rapidly. One study using alpine transects predicts between 12-28% of all plant species will be lost from their current sites in the next 50 years (Román-Palacios & Wiens, 2020). Nearly 600 plant species have officially been lost, primarily due to human activity disrupting habitats over the last 250 years; the actual number, including undescribed and unnoticed species is likely significantly higher (Humphreys *et al.*, 2019).

Recognising a plant species as extinct can be difficult for two reasons. First, finding individuals can be difficult making it hard to conclusively prove extinction (Crawley, 1990). Second, functionally extinct species, i.e. non-viable but with surviving individuals, can persist for an extremely long time. The “extinction lag” between a woody plant’s functional extinction and the death of the last remaining individual may be several centuries; temperate forest herbs may persist for 100 years after habitat fragmentation seals their fate (Kolk & Naaf, 2015). This concept of extinction lag in plants suggest many species may have already succumbed to climate change but for the fact of their fading relicts.

Climate change can cause a decrease in effective population size in plants, leading to increased risk of extinction. It can reduce effective population by population fragmentation,

mass die-offs and strong selective sweeps, reducing available genetic diversity. This reduction in genotypic diversity can lead to loss of adaptive potential and inbreeding depression, both of which reduce the long-term stability of the populations (Richards, 2000; Sork & Smouse, 2006). Maintaining genetic diversity is vital for conservation, especially in uncertain conditions where adaptive potential is paramount to survival (Aavik & Helm, 2018).

Conservation interventions may also pose a threat to plant populations if not handled correctly. Introduction of novel genotypes to inbred populations can be beneficial, as seen in positive examples of genetic rescue, but if the wrong genotypes are introduced it can cause outbreeding depression. Outbreeding depression is a loss of fitness sometimes seen in the descendants of pairings between individuals from different populations (Frankham *et al.*, 2011). There are several mechanisms of outbreeding depression, but the most relevant here is conflict of adaptive responses between populations: adaptively beneficial alleles from one population may be maladaptive when introduced to another. Improper selection of foundational stock for replanting initiatives can similarly lead to poorly adapted resultant populations, or even inbred populations (Thomas *et al.*, 2014).

1.5.2 Conservation interventions

One of the core aims of conservation work is the creation of self-sustaining populations and/or ecosystems. Self-sustaining here taken to mean long-term[6] continuation of the population without human intervention, or in the case of certain semi-natural habitats, without human intervention beyond the base standards of the habitat (e.g. grazing livestock on semi-natural grasslands). Further reaching into the definition of self-sustaining under current concerns about climate change may include the idea of "future-proofing" populations against predicted changes in climate. The actual aims in conservation work may differ between, broadly, the return to a former state or the creation of a novel state predicted to be better adapted to continuation (Coleman *et al.*, 2020). Depending on the reasons for a population's failure, attempting to return to the prior state may be ineffective

[6] i.e. indefinitely barring naturally occurring climatic shifts, community successional changes or other *expected* causes of extirpation/extinction

or undesirable – if a population has suffered due to changes in environmental conditions, reinstating the same genotypes may be setting it up for failure.

Habitat restoration aims to undo damage to an ecosystem, e.g. restoring prior hydrological conditions in drained wetlands, reintroduction of key species or genotypes and changes in grazing management (Bobbink & Willems, 1993; Godefroid *et al.*, 2011; Mälson *et al.*, 2008; Öckinger *et al.*, 2006). Reseeding is used to secure soil surfaces, reintroduce species/genotypes to a community and exclude undesirable species early in restoration (Török *et al.*, 2010). Restoring degraded habitats to a more species rich and environmentally heterogeneous state may increase community resistance to climate change[7]. Habitat creation is an overlapping concept in which conservationists attempt to recreate habitat types at novel sites with appropriate edaphic conditions (Anderson, 1995).

Genetic rescue is a, sometimes controversial, method of trying to save populations suffering the effects of low effective population size (Waller, 2015). Genetic rescue involves introducing individuals from other populations to struggling ones to bulk the numbers and introduce novel genotypes, with several high profile success stories: the Florida panther, *Puma concolor couguar*, population in the 1990s suffered high mortality rates and numerous visible genetic defects, however the release of 8 females from a related population in Texas in 1995 appears to have reversed several deleterious trends (Pimm *et al.*, 2006); an isolated population of adders, *Vipera berus*, in southern Sweden was likewise rescued by the addition of 20 males from a nearby, larger population (Madsen *et al.*, 2020). Despite these success stories, the results have occasionally been catastrophic as in the case of the Alpine ibex, *Capra ibex*, in what was then Czechoslovakia: the addition of ibex from other populations led to hybrids kidding in winter as opposed to spring, and wiping out the population entirely (Waller, 2015).

Reintroduction and conservation introduction are two related approaches that aim to create a population of a plant in a location where it is currently absent (Maschinski & Haskins, 2012). When extirpated from a site, species may be able to recolonise restored habitat from adjacent populations or the soil seedbank, however where this is not an option they can be

[7] "the ability of a community to maintain its composition and biomass in response to environmental stress" per (Grime *et al.*, 2000)

manually reintroduced. Reintroductions aim to restore species to sites within their historic range; conservation introductions use the same methods to introduce species to novel sites outwith their historic range often as a safeguard measure[8]. While there are a great many factors influencing success/failure of these methods, one of clear importance is the selection of appropriate propagule material with attention to local adaptation, mating system and long-term genetic viability of the created population (Neale, 2012).

Composite provenancing is the practice of selecting genotypes from multiple sources, casting the net wide for traits advantageous to the intended site (Aitken & Bemmels, 2016). By creating a new and diverse pool of genotypes from divergent populations, which is then subject to selection *in situ*, people hope to create a well-adapted population with good genetic diversity. More recently, and with a mind to climate change in particular, has been the idea of predictive provenancing as opposed to the more traditionally held idea that local is best. Predictive provenancing aims to select plants for reseeding/reintroduction/introduction interventions based not on assumptions of local adaptation but from locations likely to have adaptations fit for current/future environmental conditions (Breed *et al.*, 2013, 2018; Prober *et al.*, 2015). While obviously at risk from outbreeding depression due to pleiotropic effects and improper selection of source populations it still has the potential to be a powerful tool in the development of climate resilient populations of plants.

1.5.3 The importance of understanding genetic diversity in successful conservation interventions

A number of review papers of the last decade highlight the importance of population genetics in the planning of successful restoration initiatives (Aavik & Helm, 2018; Coleman *et al.*, 2020; Mijangos *et al.*, 2015; Neale, 2012; Thomas *et al.*, 2014). Considering what we know already about the effects of population genetics on long-term prospects of a population, and the failure rates seen in reintroduction projects (Dalrymple *et al.*, 2012; Godefroid *et al.*, 2011), it is evidently vital that we incorporate genetic understanding into

[8] E.g. predictive translocations based on climate change, establishing the species over a larger area in the hopes of avoiding extinction due to stochastic events and planting away from current range to avoid issues of disease as in *Banksia spp.* in Australia to avoid *Phytophthora cinnamomi*

the planning of such interventions. If we are to create self-sustaining populations they must be genetically diverse enough to persist – the definition of which varies with species

In order to do so I identify the following as important background information to inform future conservation efforts: levels of genetic variation in stable, natural populations; genetic character of source populations; the effects of propagule management on the genetic potential of derived populations; and the shape of the landscape's effects on the distribution adaptive variation. To generate genetically robust populations, we must understand what constitutes an appropriate level of genetic variation for species in healthy populations. Source populations may be more or less related to one another and, while not a straightforward process, choosing sources of genetic variation that are likely to complement each other while being distinct enough to warrant their inclusion can be highly beneficial. Propagule management prior to introduction has the potential to skew genotypes that make it through to the eventual population – genotypes that perform well under glasshouse conditions may be poorly suited to field conditions. And finally, landscape genetics can improve our understanding of the importance/levels of local adaptation for a species, and whether source populations are likely distinct or maladaptive contributions to future efforts.

1.6 Study system

1.6.1 Calcareous grasslands

This thesis focuses primarily on calcareous grassland, i.e. those on base rich bedrocks, often limestone or chalk. They are characterised by alkaline soils, and are often nutrient poor and with exceptionally high biodiversity. They are classified under the prefix CG in the national vegetation classification system (NVC); of specific interest to this project is CG2 grassland, also known as *Festuca ovina-Helictochloa pratensis* grassland.

CG2 grasslands are a semi-natural, nutrient poor grassland that once was widespread across lowland Britain. Maintained by grazing, these grasslands are typically a low turf dominated by a fine weft of interwoven *Festuca ovina* with other grasses and a high proportion of forbs. Changes in land use over the last century, particularly the use of nitrogenous fertilisers to "improve" pasture, have degraded most of our low nutrient calcareous grasslands. 83% of Britain's semi-natural calcareous grasslands have been lost between the

1930s and 2000 (Hooftman & Bullock, 2012) . Remaining CG2 grasslands are now largely protected as Sites of Special Scientific Interest (SSSIs), and primarily restricted to steeply sloped hill and valley sides.

1.6.2 Study sites

Three major study populations are used in this thesis: 1 – A collection of CG2 sites from across the Peak District, selected for either north or south facing slope aspect. 2 – The Buxton Climate Change Impacts Laboratory, a long-term climate manipulation project. 3 – Emorsgate Seeds successive generations of *F. ovina* and their source population at Grass Wood in the Yorkshire Dales.

The White Peak region of the Peak District is characterised by a limestone bedrock and deep, steep sided valleys called dales. Opposing north and south facing dale-side slopes are climatically differentiated due to the angle of insolation, creating a natural climate laboratory. The downstream effects of this angle on the climate of these opposing pairs is notably measurable in soil temperature and rate of drying. A twelve year study on one pair of slopes at Lathkill Dale found mean soil temperatures for south facing slopes was 2.4°C higher across the year than opposing north facing slopes; differences include significantly more pronounced daily highs during the summer, where highest soil temperatures recorded on south facing slopes may be 6°C above their north facing counterparts (Rorison *et al.*, 1986).

Twenty-two sites were selected within the wider Peak District, ten north facing and twelve south facing (Fig. 1.1). Limitations on availability of quality CG2 grassland on north facing slopes identified limited the number available. Wherever possible sites were paired with an adjacent or near-adjacent opposite.

The Buxton Climate Change Impacts Laboratory (BCCIL) is a long running experiment in climate manipulation based in the Peak District. The experiment consists of a west facing, CG2 grassland on a dale-side near Buxton divided into 3 × 3m plots subject to climate manipulation treatments. The climate treatments consist of soil heating, watering, drought, control plots and combined heating and drought and heating and watering, each treatment

has 5 replicates[9]. The experiment has been running for over twenty-nine years at this point, documenting community level changes in response to altered climate.

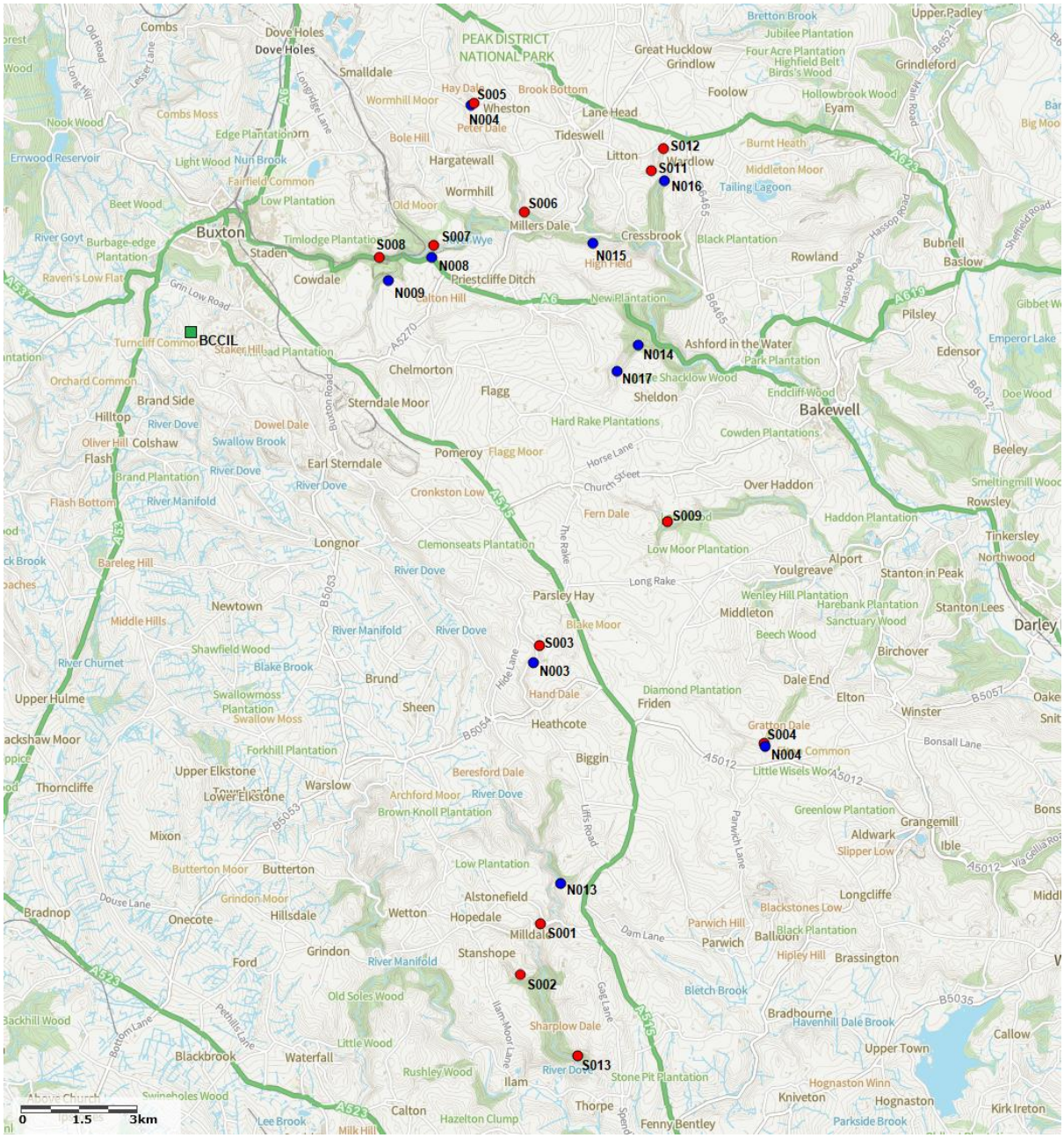

Figure 1.1 – Map of the Peak District near to Buxton showing the location of south facing (red circles) and north facing (blue circles) field sites and the BCCIL (green square)

Emorsgate seeds (http://www.wildseed.co.uk/) is a company that collects and propagate wild plant seeds for use in habitat restoration and is the CASE partner for this project. Amongst the ≈200 species they propagate is a stock of *Festuca ovina* collected from Grass Wood in Yorkshire and raised in their production system for 6 generations so far. The

[9] Treatments details: see section 1.5.4, Ravenscroft *et al.*'s work on the site.

company collects a number of wild seeds from native plants to grow in polytunnels at their sites in Norfolk near King's Lynn and near Bath in Somerset. Successive generations are raised to generate enough seed for sowing in strips in fields and harvesting for use in conservation work and landscaping. Due to their involvement in conservation work, and the discussion surrounding provenancing of seed for restoration, they are interested in the genetics underpinning climate adaptation in *F. ovina* and contributed funding to this project.

1.6.3 Festuca ovina

Sheep's fescue, *Festuca ovina*, is a short, tussock forming, cool season grass from a large, globally distributed genus in the Poaceae (tribe Poeae). One of the fine-leaved fescues, it comprises a morphologically variable species with diploid and autotetraploid sexually reproducing populations, as well as sterile triploid populations in Britain[10]. Its distribution within Britain covers much of the mainland, but with sparser presence in the east of England (Fig. 1.2). There is also the taxonomically problematic *F. vivipara*, an asexually reproducing tetraploid which likely represents a number of unrelated taxa nested within *F. ovina* (Chiurugwi *et al.*, 2010).

F. ovina is a slow growing perennial, with British populations showing exclusively intravaginal tillering, differentiating it from the morphologically similar *F. rubra* (Stace *et al.*, 1992). Its tillering pattern limits horizontal spread, though given enough time, under stable conditions, individual plants may expand to cover an area several metres across (Harberd, 1962). Data on its growth rate and the genetic testing confirming the spread of a genetic individual have been used to estimate the ages of the largest clones suggesting potential ages of 500-1000 years for some specimens, though these may be overestimates (Harberd, 1962).

Flowers are born on panicles in May-June and, like other grasses, it is wind pollinated. Seed matures in July and germinate when there is sufficient soil moisture. It is usually considered an obligate outcrossing species with strong self-incompatibility in the diploid populations, though this appears somewhat relaxed in tetraploids (Watson, 1958).

[10] Diploid, triploid and tetraploid *F. ovina* are well recorded from Britain (Watson, 1958) but accessions other parts of the range are known to vary from diploid to octoploid (Qiu *et al.*, 2020).

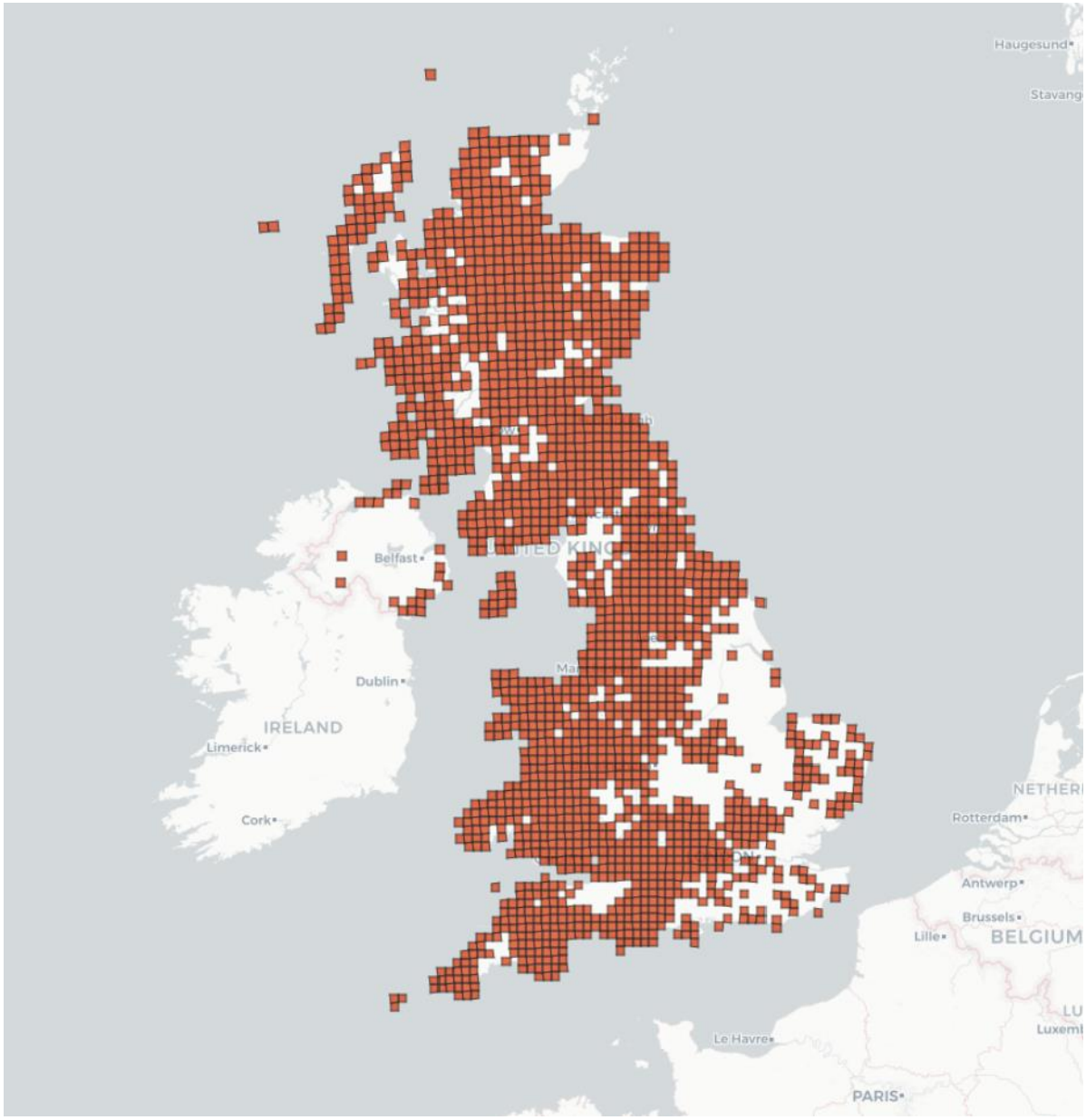

Figure 1.2 – The distribution of F. ovina in the United Kingdom, red marks species presence in 10x10km squares. Map from National Biodiversity Network (https://records.nbnatlas.org/)

1.7 Aims and objectives

Phenotypic variation of *F. ovina* populations from differing microclimates *Chapter 2*

In this thesis I will assess the signals of evolutionary differentiation due to microclimatic environment using *F. ovina* from the Peak District. Observed differences in morphology of *F. ovina* on north and south facing slopes has been attributed to both climate adaptation and

plastic responses to climate. As climatic adaptations in plants is often observable in their morphology, I will analyse morphological differences from wild collected plants grown under common garden conditions for signs of microclimate driven selection.

The influence of microclimate on the genetic structure of *F. ovina* populations *Chapters 3 and 4*

The Peak District sites represent old populations subject to consistently differing climatic effects over hundreds of generations. The BCCIL plants represent populations that have undergone 25 years of consistent climate manipulation, at the time of sampling, for a single climatic variable. By characterising this I hope to increase our understanding of the adaptive potential of these populations, and identify loci under selection for climate adaptation.

The effects of commercial propagation on environmentally adaptive genetic variation *Chapter 5*

Emorsgate Seeds, the industrial partner for my project, propagate seeds from wild plants for use in ecological restoration. Of importance to them and their customers is the creation of a robust and self-sustaining population of the species they provide once planted in different environments. Conditions in the fields are markedly different from many of the habitats seed is destined for and may be undergoing selection for traits that will be maladaptive in the wild. Having identified loci under selection for climate using wild populations and the BCCIL samples I will look for generation-on-generation changes in allele frequencies at these loci for their current line of *F. ovina*, testing whether there appears to be any loss or skew in allelic diversity, as well as searching for signs of selection for cultivation.

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2 Genetic differentiation in plant phenotype between slope-aspect microclimates in the grass *Festuca ovina*

2.1 Abstract

Climate change, including rises in global temperature and changes to weather patterns is predicted to negatively impact many plant populations, driving evolutionary and genetic changes (Román-Palacios & Wiens, 2020; Sales *et al.*, 2020; Suggitt *et al.*, 2018). While climate impacts through time and at biogeographical scales are relatively well studied, localised microclimatic variation within landscapes may be crucial for shaping plant adaptation and genetic structure. Understanding the extent to which plant populations are adapted to microclimatic variation is vital to understanding their contribution to adaptive potential at the landscape scale and in determining the fate of populations during climate change. Here, I used north and south facing slope aspects as a study system to investigate whether landscape-scale microclimatic variation drives evolutionary differentiation in plant phenotypes, in the grass *Festuca ovina*. North and south facing slope aspects are an important source of microclimatic variation. The environmental heterogeneity they provide may act as a force to maintain climatically adaptive genotypes, which may be expressed in morphological phenotypic differences between populations occupying different microclimates. I collected *Festuca ovina* plants from eight populations occupying cool and moist north facing slopes and eight populations occupying warm and dry south-facing slopes in the Peak District National Park, UK. The collected plant material was grown in a common garden experiment at Ness Botanic Gardens over six months, and plant phenotypes were measured. I observed significant differentiation in plant phenotype between *F. ovina* populations originating on north- and south-facing slope aspects. My results show that north and south facing slope-aspect microclimates favour different morphotypes of *F. ovina* for both vegetative and reproductive traits. Plants were significantly differentiated at the slope aspect level for plant height, leaf length, reproductive effort and leaf tissue density. Plants originating from north facing populations invested more in taller growth and greater allocation to vegetative growth, whereas those from the warmer, drier south-facing populations made a greater relative investment in sexual reproduction. I also observed significant phenotypic variation among populations that was not related to slope aspect.

Given that they were observed in a common garden environment, the microclimate-associated phenotypic differentiation that I have documented is likely to be genetic (or perhaps epigenetic) in nature, although persistent environmental effects on the phenotype cannot be ruled out. The documented phenotypic differentiation may reflect prevalent selection pressures acting in the different microclimatic niches. More intense competition in cool moist microclimates would require a greater investment in plant growth relative to reproduction, in order to maintain competitive ability. Conversely, in south-facing slope aspects, harsh abiotic conditions may be the most important determinant of plant fitness (via drought and high temperatures) selecting for reproduction as a strategy to escape from environmental stress.

2.2 Introduction

Evolutionary change encompasses heritable changes in plant phenotypes and genotypes within populations arising from adaptive and neutral processes (McCoy, 2018). Genetic differentiation between populations is driven by selective pressures and reproductive isolation, and eroded by gene-flow (Antonovics, 1968; McNeilly, 1968; McNeilly & Antonovics, 1968). Selective pressures that differ between populations will favour the spread of different alleles while reproductive isolation amplifies the ability of these populations to become differentiated. Geographically distant populations are expected to be genetically different for reasons of both genetic drift and differential selection pressures, while any differing selective pressures acting on adjacent populations must overcome gene-flow if the populations are to show evolutionary changes.

Edaphic conditions — i.e. soil properties, including composition, physical structure and nutrient availability — are a long-studied source of selection pressure driving evolutionary differentiation between plant populations (Antonovics, 2006; Bradshaw & Snaydon, 1959; Prentice *et al.*, 2015; Snaydon & Davies, 1972). An early study demonstrated geographically distant populations of *Festuca ovina* from acid and calcareous (basic) grasslands had different levels of calcium tolerance, as measured by total dry weight (i.e. net C assimilation) of plants grown in sand culture with 0, 5, 10, 20 and 100ppm calcium in the watering solution. Plants from high calcium soils performed better with higher calcium availability than with less, and vice versa (Bradshaw & Snaydon, 1959). These results demonstrate

innate, differential responses to environmental factors between the populations, i.e. evolution. Later work with *A. odoratum* populations from the Park Grass experiment, a long running (166 years) experiment in fertiliser application to pasture, at Rothamsted demonstrated that liming and fertiliser application (applied since 1903 and 1856 respectively) was able to drive adaptive differences between populations (Snaydon, 1970), with the evolutionary response of populations to altered edaphic conditions identifiable as morphological differences under constant culture conditions, hence, genetic in nature (Snaydon & Davies, 1972). Heavy-metal contamination is a very strong selective pressure, often fatal to non-tolerant plants, with highly localised effects. Sites with sharp boundaries between heavy-metal contaminated soils and non-contaminated soils have provided some important study systems, such as in the works of Bradshaw and Antonovics on the Trelogan lead mine, Flintshire (Antonovics & Bradshaw, 1970). Earlier root-growth studies had already demonstrated that *Agrostis tenuis* plants from the uncontaminated pasture were not lead tolerant, unable to form roots on lead-contaminated soils, while those from lead contaminated soils grew without issue (Jowett, 1964). They used *Anthoxanthum odoratum* plants collected from a transect covering lead-and-zinc-contaminated soil from the mine and immediately adjacent uncontaminated soil from an adjacent pasture. These populations were differentiated in their plant height, flag-leaf length, self-fertility and culm length. This was in addition to established differences in metal tolerance and flowering time, in which the metal tolerant population flower earlier than the normal plants (Antonovics & Bradshaw, 1970; McNeilly & Antonovics, 1968). The nature of these studies, observing differences between populations between conditions *in situ* and grown reciprocally exposed to the relevant environmental differences helped demonstrate that, with a significant selection pressure, plant populations can undergo extensive differentiation in relatively short spatial and temporal scales.

Climate is a strong selection pressure that is well known to influence plant communities and populations (Bemmels & Anderson, 2019; Hoffmann & Sgrò, 2011; Jump & Peñuelas, 2005; Macel *et al.*, 2007; Ravenscroft *et al.*, 2014; Trinder *et al.*, 2020). Space-for-time substitutions are an approach in ecology commonly used in regard to climate, to studying temporal processes (e.g. evolution, succession, impacts of climate change) by using sites with differing characteristics as a proxy for time-points (Blois *et al.*, 2013). Alpine or

montane study systems, primarily their altitudinal gradients, have been used as a space-for-time system to investigate the effects of climate and microclimate (local scale climatic differences such as those caused by topology and insolation) on plant populations (Giaccone *et al.*, 2019; Lampei *et al.*, 2019; Oldfather & Ackerly, 2019; Opedal *et al.*, 2015). Space-for-time uses the climate differences in space as a proxy for climatic changes over time — elevation can form an easy source of climatic variation, however, is not a perfect system. A common garden experiment using *Arabidopsis thaliana* from the Italian Southern Alps found that altitude was a poor predictor for frost-hardiness compared to microclimate (number of frost days), demonstrating microclimate's importance in shaping adaptive response in plants (Lampei *et al.*, 2019).

Climate manipulation studies work by creating distinct, repeatable microclimate patches and monitoring the changes that occur as a result. One such experiment is the Buxton Climate Change Impacts Laboratory (BCCIL), which is an old sheep pasture on a west-facing slope. Work there has shown both genetic (Ravenscroft *et al.*, 2015; Trinder *et al.*, 2020) and morphological changes (Ravenscroft *et al.*, 2014) in forbs and grasses in response to 15+ years of manipulation — climate treatments consist of heating, watering, drought and control plots, as well as combined heating + watering and heating + drought. The work by Trinder *et al.* (2020) demonstrated heritable differences in early-acting life history traits in *F. ovina*, amongst progeny of an F1 array using parent plants from drought and control treatments, giving strong evidence that observed changes are genetically controlled, i.e. evolutionary. Ravenscroft *et al.* (2015) demonstrated strong, consistent, signals of genetic differentiation in *Plantago lanceolata* and *F. ovina* between climatic treatments and control plots. Climate manipulation studies focus on particular climate variables rather than topographical patterns, which is highly informative but not strictly representative of natural systems due to lack of covariance of variables.

Topographical slope aspect is a common source of local-scale microclimatic variation used in space-for-time studies and can drive significant differences between north and south facing slopes (Rorison *et al.*, 1986). Local-scale microclimate has been shown to act to provide climatic refugia for plants under threat from climate change in alpine and riparian woodland ecosystems (Ellis, 2020; Opedal *et al.*, 2015). Unlike latitude and altitude as sources of climatic variation, the more similar base conditions of slope-aspect microclimates (rainfall,

wind, seasonality) and physical proximity of their populations can make them more directly comparable. A large body of work has been dedicated to one particular slope-aspect study system, the “Evolution Canyon” (EC) sites in Israel (Nevo, 2012). The EC consists of four valleys, each on an approximately east-west axis with a south facing “African” slope and a north facing “Eurasian” slope. *Hordeum spontaneum* populations from opposing slopes at EC have demonstrated differences in copy number of the BARE-1 transposon, with south facing slopes having more (Kalendar *et al.*, 2000). Flowering time in *Ricotia lunaria* at EC was differentiated for slope-aspect, with genetic differentiation between the populations supported by altered allele frequencies (Kossover *et al.*, 2009). The work from these valleys has been highly informative about population differentiation at small spatial scales driven by the microclimate. However, most work in this system has restricted itself to a single canyon, all four of which show significant differences between the vegetation type for both “African” and “Eurasian” slopes (Nevo, 2012). Furthermore, the EC study system is notable for the extreme nature of the difference between slope aspects, which is both a strength of the system and a weakness: opposing slope aspects in temperate Europe do not show this level of climatic differentiation.

Current studies using slope-aspect microclimates tend to be limited in the level of replication of sites with different microclimates, with many studies investigating only a single pair of slope aspect sites. This lack of replication makes it hard to firmly attribute observed differences between populations to climate — sites will differ for other abiotic conditions, and any two isolated populations are expected to show differences in some phenotypes regardless of environment. With replication you can identify whether a trait is truly associated with climatic conditions. Evidence suggest microclimate may provide sources of climatically adaptive genetic variation that buffers plant communities against climate change (De Kort *et al.*, 2020; Suggitt *et al.*, 2018), there is a need for more studies focusing on slope-aspect microclimate and evolutionary differentiation, that conclusively evaluate the role of natural microclimatic conditions in driving population differentiation. As such, studies are invaluable in informing conservation approaches to safeguarding plant populations against climate change (Ellis, 2020; Oldfather *et al.*, 2020; Suggitt *et al.*, 2018). Furthermore, other space for time microclimate studies investigate extreme differentiation in climate conditions that support wholly different vegetation types, with the resulting

changes in the biotic environment potentially obscuring or confounding evolutionary responses (Damgaard, 2019). Overall, there is a lack of research that focusses on microclimatic variation at local spatial scales, and which is not extreme in its extent. As a result, we lack knowledge on how typically occurring microclimatic conditions may influence the adaptive potential of populations with regard to climate (Balkenhol *et al.*, 2017; Byers, 2005), and the implications of this for adaptive responses to future climate change, which is a pressing issue for ecological conservation and restoration (Mijangos *et al.*, 2015).

In this chapter I will address the question of whether microclimatic selective pressures drive evolutionary differentiation in plant phenotype, using natural populations of the grass *F. ovina* in a well-replicated, spatial mosaic of north and south facing topographical slope aspects and their associated contrasting climatic conditions as a study system. In light of the strong evidence that populations may be evolutionarily differentiated with regard to microclimate (Lampei *et al.*, 2019; Ravenscroft *et al.*, 2015), and over short spatial scales where there is persistent gene-flow between populations (Antonovics & Bradshaw, 1970; McNeilly, 1968; McNeilly & Antonovics, 1968), it seems plausible that north and south facing slopes in the Peak District may harbour consistently differentially adapted populations. To investigate this, I use plants from 16 populations in the Derbyshire Dales—representing eight north and eight south facing slopes within a 26x11 km area—by growing them under common environmental conditions to identify whether specific traits are associated with microclimate. If population mean phenotypes are found to associate with microclimatic conditions in this spatially replicated study system, then it would provide support for the hypothesis that these populations have undergone microclimatic selection associated with slope aspect. Though all slopes differ, using replicated north and south facing slopes within a relatively small area, where bedrock chemistry and the overarching climate remain constant, allows for the identification of effects caused by slope-aspect microclimate.

I show that a number of plant traits, including plant height, leaf length and tissue density, are consistently differentiated between north and south facing slope aspects. Plants from north facing slopes were, on average, taller and with longer, denser leaves than south facing plants. South facing plants however showed greater average investment in flowering than those from north facing slopes. Other traits, e.g. tiller number, show significant among-

population variation but with no overall difference with slope aspect, suggesting that other non-climatic factors are also driving evolutionary differentiation in this study system.

2.3 Methods

In order to investigate the effect of genotype on the morphology of populations of *Festuca ovina* a common garden experiment was set up at Ness Botanic Gardens, Merseyside, UK (SJ 30291 75576). The plants used were collected from a series of slopes in the Peak District.

2.3.1 Study system

As the study species I used the grass *Festuca ovina*, a morphologically variable species (Stace *et al.*, 1992) distributed over an extensive range of habitat types and latitudes. The morphology of *F. ovina* has been studied in depth since the first half of the 20th century, including in relation to geographic and climatic gradients. One early study on *F. ovina* populations from across Scandinavia demonstrated heritable differences in spikelet size on a gradient from north to south, and regional differences in drought tolerance (Turesson, 1926). Watson (1958) investigated ploidy levels in British populations of *F. ovina sensu lato*, and found that plants morphologically identified as *F. tenuifolia* in the field would often resemble *F. ovina* under greenhouse conditions, showing that morphology may be plastic with regard to environmental conditions. However, an altitudinal transect of plants from Ben Cruachan (0-1124m, every ≈61m) grown under constant culture conditions showed altitude of collection to be a significant predictor of both leaf length and width – thus demonstrating heritable morphological differences at a fine spatial scale in response to environment (Watson, 1958).

The sixteen sites used (table 2.1) were selected from across the Peak District National Park, and are a subset of the larger network of sites surveyed for genetic work (see chapter 3.). Criteria for the selection of sites included being good quality CG2 grassland (Rodwell & Pigott, 1992) and occurring on slopes with an orientation no more than 15° off north or south.

Table 2.1 – Site codes, names, slope aspect and Ordnance Survey 10-figure grid-references describing the location of the sites used in this study, see Fig. 1.1 for map view.

Site	Site name	OS grid ref.	Aspect
N004	Gratton Dale	SK 20081 59680	N
N006	Hay Dale	SK 12315 76480	N
N008	Chee Dale	SK 11341 72504	N
N009	Topley Pike	SK 10031 71868	N
N014	Deep Dale - 1	SK 16756 70113	N
N015	Priestcliffe Lees	SK 15616 72949	N
N016	Cressbrookdale	SK 17410 74548	N
N017	Deep Dale - 2	SK 16819 70189	N
S001	Mill Dale	SK 14244 54992	S
S002	Hall Dale	SK 13537 53634	S
S004	Gratton Dale	SK 20057 59746	S
S005	Hay Dale	SK 12385 76537	S
S006	Monk's Dale	SK 13753 73686	S
S008	Wye Dale	SK 09887 72514	S
S012	Cressbrookdale	SK 17305 75391	S
S013	Stepping Stones	SK 15120 51516	S

2.3.2 Collection of living plant material

Twenty-two plants were collected from each site, along a 1x10m grid, collecting the nearest mature *F. ovina* plant to each grid vertex (Fig. 2.1). All sampled plants had a minimum of 10 tillers which were all physically connected to each other, to ensure tillers were all derived from the same plant genotype (genet).

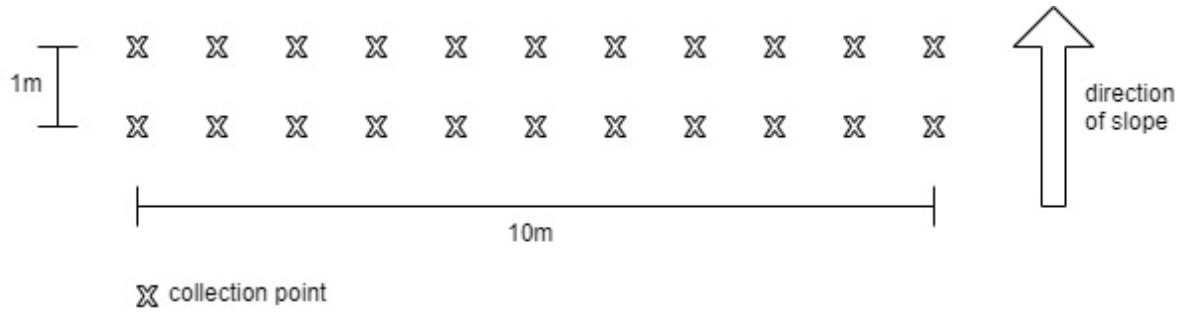

Figure 1.1 - Sampling pattern for plants, a horizontal block across each slope of width 10 metres and height 1 metre. Samples were taken at 1 metre intervals along the top and bottom of the grid, marked here with Xs.

Each plant removed was transferred immediately to a 7.5x10cm sealable polythene bag containing a pre-marked planting label. Bags also contained a small rectangle of folded blue-roll, moistened with deionised water, to prevent plant dehydration. Plants were collected over the course of 3 days and stored in a cool, dark place until planting.

2.3.3 Experimental setup and maintenance

Collected plants were planted in square 7x7x9cm pots (Fig. 2.2.), in a 1:2:1 mixture of perlite, John Innes No. 1, and natural rendzina soil (collected at a site adjacent to the experimental plots at the BCCIL). Experimental groups (populations) consisted of 20 of the 22 plants collected per site, plants were standardised to 10 tillers and a leaf canopy height of 2.5cm at planting. All individuals were arranged in a fully randomised design (Fig. 2.3.), in trays of 15, across two bays. This was done so as to minimise positional effects influencing subsequent growth patterns. Pots were labelled with plastic plant labels marked in pencil recording the unique ID of each individual.

Plants were grown for 6 months (June 2021-January 2022) before data collection began. During the months of June-September, pots were maintained in a well-watered state, with watering schedule dependent on rainfall. At least once monthly during the active growing season, weeds were removed from pots to avoid effects from competition.

Figure 2.2 - A tray of F. ovina *plants moved for tiller counting, with one removed, displaying arrangement of pots in a tray and labelling system employed (Jan 2022)*

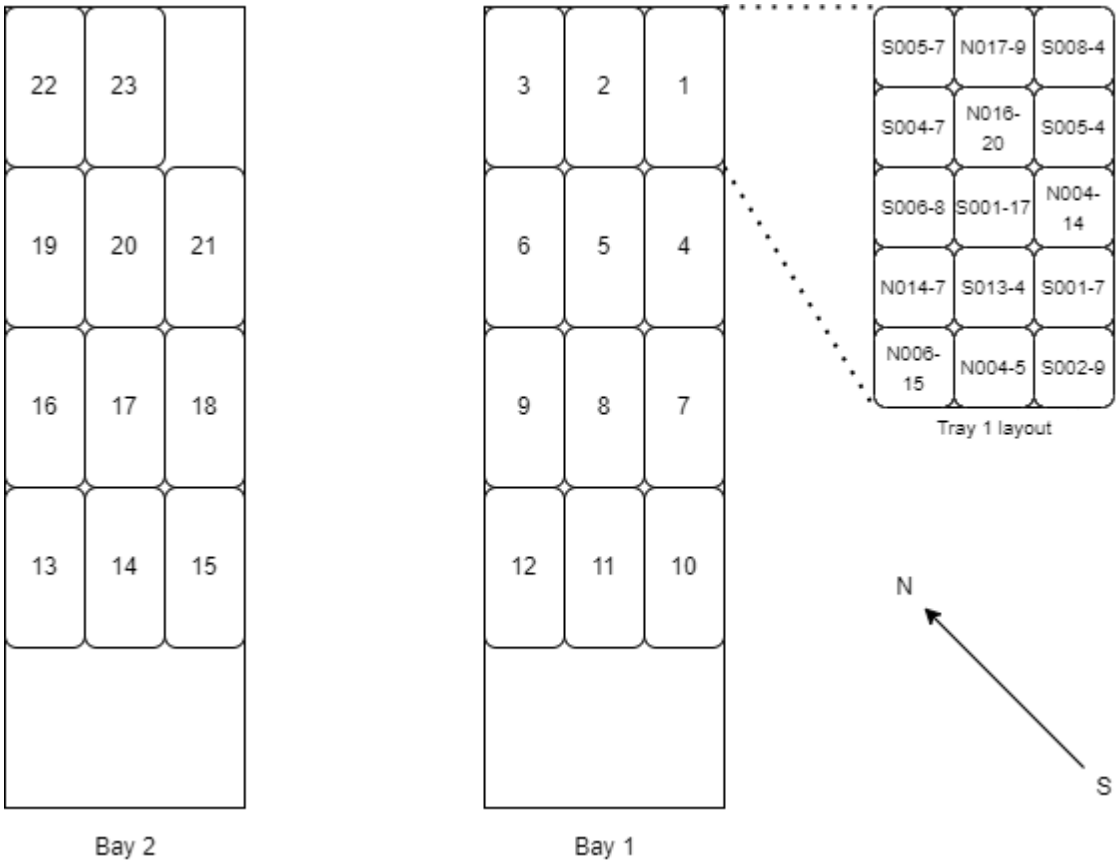

Figure 2.3 - Layout of trays in experimental setup, consisting of raised wooden bays at Ness Botanic Gardens, Neston, with example layout of individual pots within a tray. Blocks marked 1-23 are trays; squares marked S005-7 etc. are individual pots.

2.3.4 Trait measurements

I measured the following traits for each experimental plant: tiller number, plant height, leaf length and width, wet and dry single leaf mass, above ground biomass, specific leaf area and number of flowering tillers. Traits were chosen for their known or suspected associations with environmental adaptations or genetic variation.

Tiller number: The number of tillers produced by healthy *Festuca* plants from standardised clones under standard conditions is known to have a genetic element, making it a valid target for investigating differentiation (Harberd, 1962). Further to that, the trait might be expected to covary with other traits such as leaf length and plant height under conditions of equal growth, and vegetative tiller number influences the potential number of flowering tillers that can arise from a plant. Field observation during collection of plants also suggested higher tiller numbers were associated with more drought-prone sites.

Plant height and leaf length: Plants growing on sites with a deeper bryophyte or shrubby layer, usually damper sites, display notably elongate phenotypes *in situ*. Whether this is a plastic response to light and/or water availability or a genetically fixed trait is unknown. Under conditions of equal total growth, they may be expected to negatively correlate with tiller number, i.e. fewer, longer tillers vs many, shorter ones. Both traits are measured independently as growth form may differ between upright and sprawling growth-patterns.

Leaf width: Leaf width was primarily measured for its importance in calculating leaf area, specific leaf area (SLA) and tissue density. SLA variation has been shown to correlate with survivorship under differing conditions of light availability and to be associated with drought tolerance, both traits known to vary with slope aspect (Wellstein *et al.*, 2017; Zirbel & Brudvig, 2020)

Wet and dry mass: Moisture content of leaves and material investment in leaves are established morphological variables associated with environmental adaptation to drought in other plant functional groups such as tree species (Ramírez-Valiente *et al.*, 2017; Wolfe, 2017).

Number of flowering tillers: Flowering tiller number has been demonstrated as a trait under genetic control and associated with climatic selection pressure for *F. ovina* plants from the BCCIL (Trinder *et al.*, 2020). Counted tiller numbers can be expressed as three separate

traits describing investment in sexual reproduction; the absolute tiller number, the proportion of vegetative to reproductive tillers (relative reproductive effort) and as a binary trait of flowering/not-flowering.

After 6 months of growth, measurements were simultaneously taken for plant height and tiller number. Each plant was taken and the number of visible tillers present was counted. Due to the intravaginal nature of tillering in *F. ovina* a tiller was defined by the presence of a visible leading leaf, to ensure standard tiller counts. To ensure repeatability of this trait measurement, the first 45 plants counted were recounted at the end, and found to be identical. Plant height, from the soil surface to the height of the tallest living leaf, was measured using a steel rule. Leaves were not stretched upright but measured at their natural maximum height to account for natural differences in growth habit. All tiller counts and height measurements were completed over the course of 2 days in January 2022.

At the end of January 2022, the longest healthy, living leaf from each plant was selected by visual inspection and harvested for leaf-length, width and wet and dry weights. Sampled leaves were cut as close to the base as possible using dissecting scissors and had no signs of senescence, less than 2mm of leaf tip burn and were not from a reproductive tiller. Reproductive tillers were identifiable through changes in morphology compared to vegetative tillers, namely a distinctive thickening and flattening of the tiller base. Harvested leaves were immediately placed in individual, labelled, 7.5x10cm sealable polythene bag with a pre-moistened rectangle of folded blue roll. Bags were placed in a fridge at 4°C for 48 hours to allow leaves to reach full turgidity before measurement. To ensure ample processing time for all samples without degradation of leaves, these measurements were split into two rounds, taking place four days apart.

Once leaves were fully hydrated they were photographed using a Canon 700D EOS camera attached to a clamp stand, on white paper next to a steel rule for scale. Each leaf was photographed twice, once laid flat (allowing measurement of length) and once pinned so that the flat leaf surfaces were oriented horizontally (to measure leaf width). Measurements were taken using the program IMAGEJ, using the measurement and segmented line tool, taking a measurement of the ruler (between two points marking 1cm) for scaling, as well as measuring 1mm on the ruler as a test of accuracy. Width was measured at the leaf mid-point.

After photography, each leaf was placed in an individually labelled paper envelope until all leaves for an experimental tray (15 in total) had been processed. Subsequently, leaf wet mass was determined on a 5-point balance (Ohaus EX225D) to the nearest 0.01mg. Envelopes were then placed in a drying oven at 60°C and dried to a constant mass. Dried leaves were then reweighed to assess leaf dry mass and leaf tissue density was calculated by leaf dry mass/wet mass. As leaf width for *F. ovina* is approximately consistent along the length of the leaf and leaves are folded in half along the midrib, leaf area was calculated as 2*leaf length*leaf width. Specific leaf area (SLA) was calculated as leaf area/leaf dry mass.

After other morphological traits were recorded, biomass was harvested in March 2022 by cutting all plants to 13mm height above soil surface and placing harvested material in individually labelled paper envelopes. Material was dried to a constant weight at 60°C. Material was weighed on an Ohaus EX225D 5-point balance.

In May 2022 flowering tillers had extended and were fully visible. The number of flowering tillers (a measure of plant reproductive effort) was assessed for each plant, and counts were checked twice. Flowering tiller number was used (i) as an absolute measure of reproductive effort, (ii) as a measure of reproductive effort relative to vegetative resource allocation (the percentage of tillers counted in February that were reproductive) and (iii) as a binary trait in the form of flowering vs. non-flowering individuals.

2.3.5 Statistical analysis

All statistical analysis was performed using the statistical software R 4.1.0 (R Core Team, 2020) using the packages MCMCGLMM (Hadfield, 2010), LME4 (Bates *et al.*, 2015) and MASS (Venables & Ripley, 2002).

The trait data were analysed to assess phenotypic differentiation between populations of north and south facing slope origin and among populations, by fitting a Bayesian Markov chain Monte Carlo generalised linear mixed model (MCMCglmm). Each trait was used as a response variable. Slope aspect and experimental bay were set as fixed effects predictors, with the latter fitted as a centred predictor (van de Pol & Wright, 2009) since I was not interested in the parameters for any specific bay, but a notional average bay. Planting tray and population were set as random effects. Since MCMCglmm fits generalised linear models, it allows for normally, categorical, binary and Poisson distributed errors without

manual transformation of the data. Models were run for 1,000,000 iterations, with a burn-in of 200,000 and thinned to every 100 iterations (=8000 iterations to form posterior distributions). Tiller number and number of flowering tillers, as count data, used the Poisson family; plant height, leaf length, leaf width, leaf area, SLA, tissue density, proportion of flowering tillers and biomass used the Gaussian family; flowering (binary) used the categorical family. Priors were inverse Wishart distributions, chosen to be relatively uninformative so as not to bias the results, giving equal weight to explanatory variable and the random effects. Variance was set to $1x10^{-10}$ and belief parameter of -1 for most analyses, with a corresponding prior set for random effects. For the binary trait of flowering, R variance was fixed at 1.

Example MCMCglmm model code for tiller count by slope aspect:

```
prior1 <- list(R=list(V = 1e-10, nu = -1), G = list(G1 =
list(V = 1e-10, nu = -1),G2 = list(V = 1e-10, nu = -1)))

mcTil <- MCMCglmm(Tillers ~ Slope + Bay.c, random = ~ Tray +
Site , data = TilLenFull, family = "poisson", nitt = 1000000,
burnin = 200000, thin = 100, verbose = F, prior = prior1)
```

The same process was applied to analyse results by site of origin, however slope was not included as a fixed or random effect, and population (“Site”) was used as a fixed effect.

Example formula for plant height by site:

```
prior2 <- list(R=list(V = 1e-10, nu = -1), G = list(G1 =
list(V = 1e-10, nu = -1)))

mcHeight.site <- MCMCglmm(Height ~ Site + Bay.c, random = ~
Tray , data = TilLenFull, family = "gaussian", nitt =
1000000, burnin = 200000, thin = 100, verbose = F, prior =
prior2)
```

Post-hoc testing to assess the difference between posteriors for the by-site models was done through comparison with a model run without site or slope as an explanatory variable (formula *response ~ 1 + Bay.c*). In addition to the use of the univariate MCMCglmm models, MANOVA was performed using the R function manova(), to assess multivariate variability among sites and slope aspects.

A principal component analysis (PCA) was performed using the MASS function princomp(), in order to investigate whether combined trait measurements grouped by site or slope aspect under reduced dimensionality. The variables passed to the PCA were tiller count, plant height, leaf length, leaf area, SLA, number of flowering tillers, above-ground dry biomass, leaf tissue density and proportion of tillers flowering. Linear discriminant analysis (LDA) was run similarly, in order to identify relationships between combinations of traits in differentiating slope-aspect populations.

2.4 Results

The morphological analysis and modelling revealed significant differences between plants at both the slope-aspect-microclimate and population level. Slope aspect microclimate and site (population) exerted an effect on growth traits (MANOVA with slope and site as factors; slope F = 32.3, *P<0.0001*; site F = 4.6, *P<0.0001*), tissue traits (slope F= 6.6, *P=0.0015*; site F = 1.7, *P=0.0175*) and reproductive traits related to flowering (slope F = 10.7, *P<0.0001*; site F = 2.71, *P<0.0001*). North-facing plants overall favouring a sparser, longer/taller growth form and lesser investment in flowering tillers compared with south-facing plants.

2.4.1 Growth traits

Significant differences were found between north- and south-facing plant populations for the traits of height, leaf length and biomass above 13mm plant height, however not for tiller numbers. South-facing plants had a greater number of tillers than north-facing plants, with posterior means of 22.5 and 20.7 tillers respectively, albeit with low statistical support (Fig. 2.5. (a)). South-facing plants were significantly (pMCMC = 0.0035) shorter than north-facing (Fig. 2.5. (b)), in line with field observations of growth habit differences between the microclimates. Longest leaf length was positively correlated with plant height, linear

regression indicates that this relationship is stronger in plants from north-facing slopes than those from south-facing slopes (Fig. 2.4.), with north-facing plants having longer leaves (Fig. 2.5. (c)). As may be expected, due to the lack of difference in tiller number with significantly greater tiller and leaf length, north-facing plants had 1.3 times the biomass above 13mm in height than south-facing plants (Fig. 2.5. (d)).

MANOVA was conducted, setting the traits of tiller number, plant height, leaf length and biomass as the response variables, and site and slope as factors, found slope to be insignificant in explaining the variance for tiller number (F = 3.84, *P=0.051*), which was better explained by site (F = 7.95, *P<0.0001*), in keeping with the results of the MCMCglmm. All other traits were found to be significantly affected by both slope aspect and site identity.

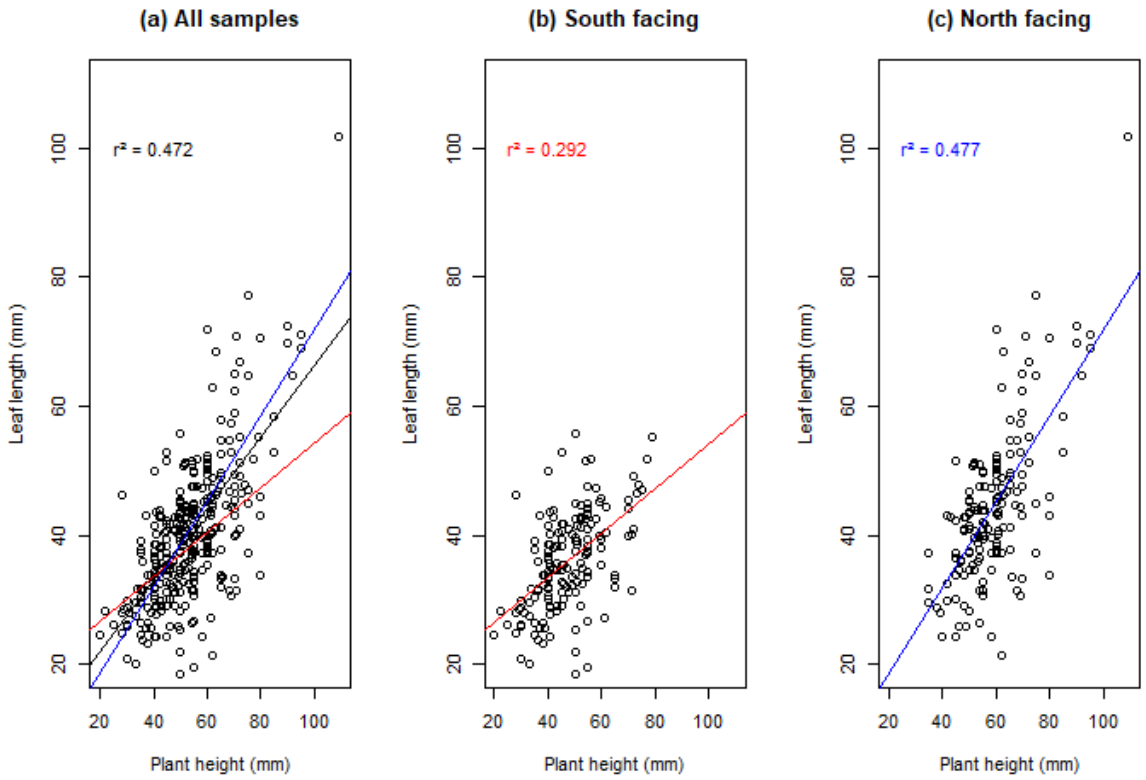

Figure 2.4 – Linear regression of measured plant height to leaf length for (a) all samples, (b) only south-facing samples and (c) north-facing samples after 6 months of growth in a common garden. Leaf length had a stronger correlation with plant height for the phenotypes found on north-facing slopes (r^2 = 0.477) than for south-facing plants (r^2 = 0.292). r^2 for all samples together was 0.472.

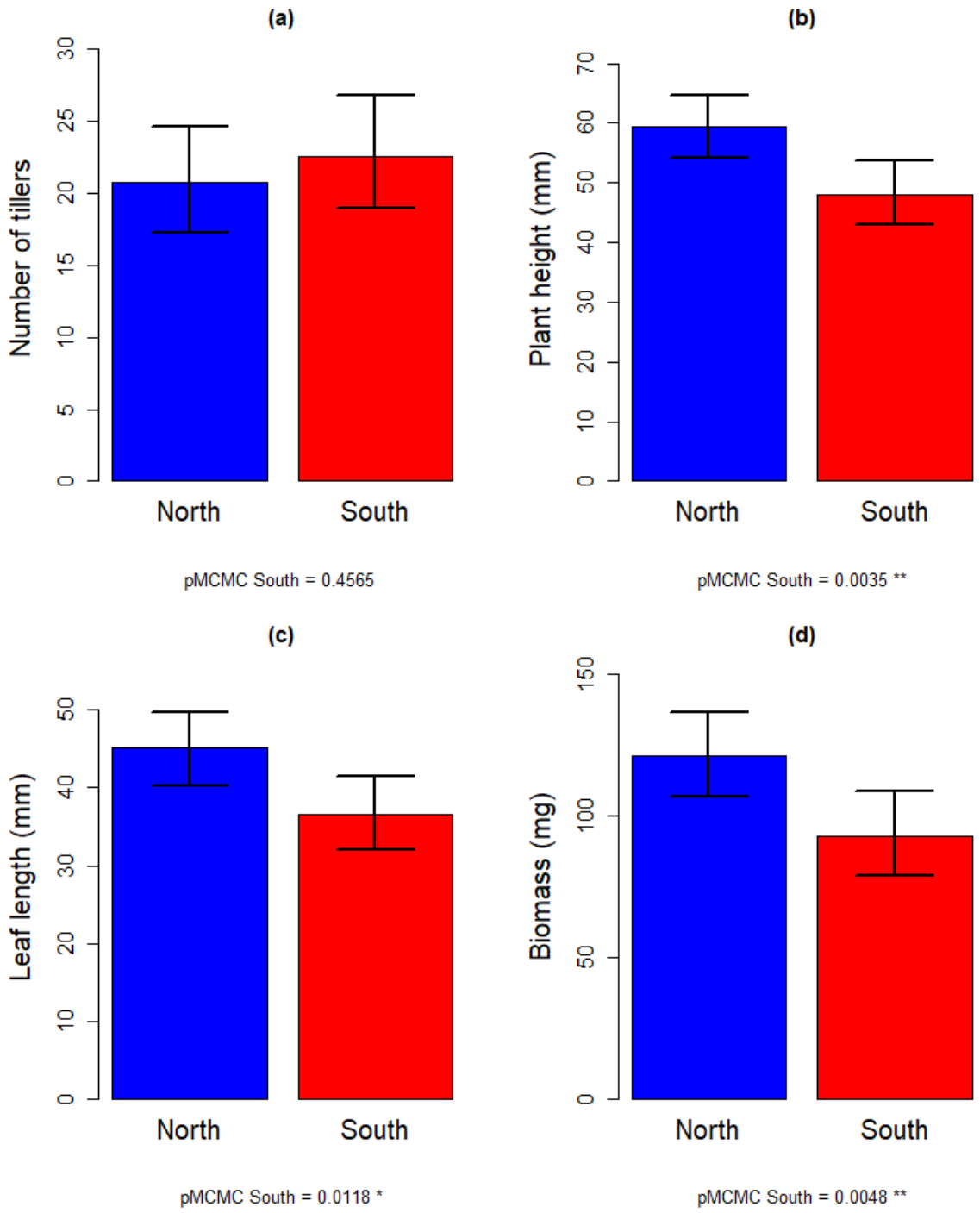

Figure 2.5 –Posterior means of MCMCglmm models for plants grown from collected from north- and south-facing slope-aspect microclimates. Plants were standardised to 10 tillers and a height of 25mm at planting and grown in a randomised fashion in a common garden at Ness Botanic Gardens for 6 months before measurement. Error bars were generated from HMD interval and represent the 95% credible intervals derived from the MCMC models. (a) number of tillers per plant; (b) plant height above ground; (c) length of longest leaf; (d) dry biomass harvested above 13mm height.

At the by-site level, tiller count, height, leaf length and biomass were all highly variable within and between groups, as shown in Figures 2.6-2.9. Tiller number analysed at the site level showed a great deal of variation between sites, with posterior means ranging from 14.21 (site S002) to 33.17 (site S001). S001, S002 and S006 had means that fell outwith the 95% HMP credible interval for south-facing sites as a whole; S001 above it and S002 and S006 below. N006 and N016 were similarly above the 95%CI for north-facing plants, and N009 below it. Plant height was negatively correlated with tiller number (Pearson's product-moment correlation: -0.364, *p<0.0001*), and positively with biomass (Pearson's product-moment correlation: 0.450, *p<0.0001*). No growth traits measured at the site level showed clear alignment into groups of north and south sites.

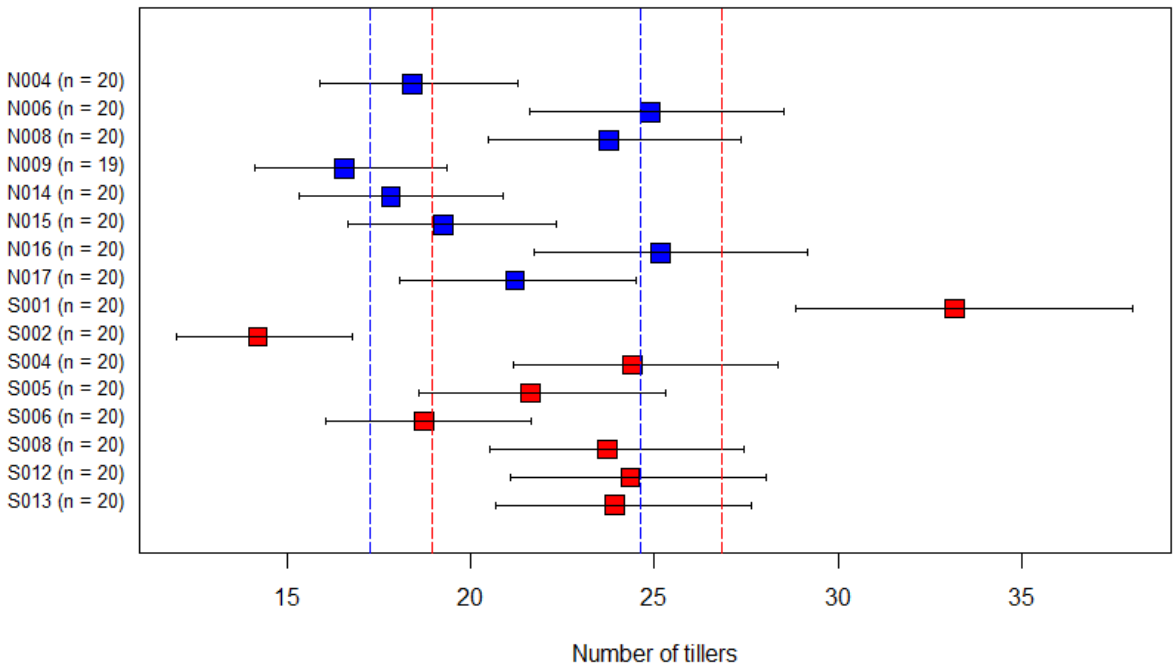

Figure 2.6 – Posterior means of MCMCglmm models (squares) and 95%CIs (horizontal error bars) for tiller counts of plants grown from collected from sixteen sites from eight north- (blue) and eight south-facing (red) slope-aspect microclimates after six months of growth. Error bars were generated from HMD interval and represent the 95% credible intervals derived from the MCMC models. Dashed blue and red lines represent the lower and upper 95%CIs for the posterior means of the MCMCglmm models, as presented in Fig. 2.5. (a).

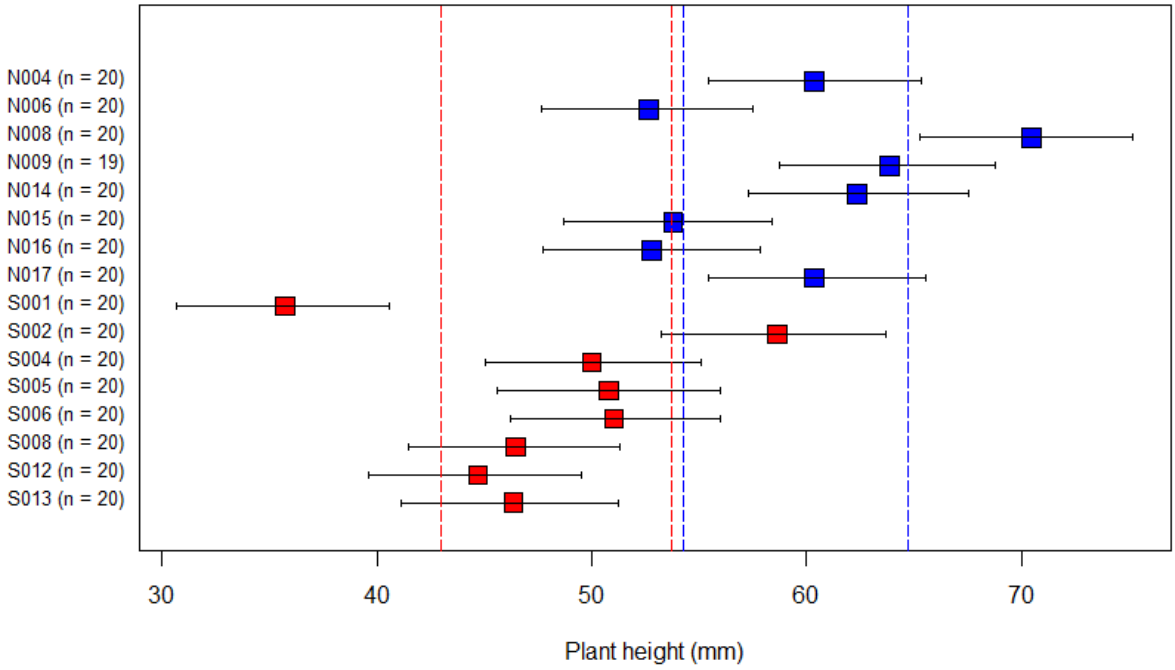

*Figure 2.7 – Posterior means of MCMCglmm models (squares) and 95%*Cis *(horizontal error bars) for height of plants grown from collected from sixteen sites from eight north- (blue) and eight south-facing (red) slope-aspect microclimates after six months of growth. Error bars were generated from HMD interval and represent the 95% credible intervals derived from the MCMC models. Dashed blue and red lines represent the lower and upper 95%*Cis *for the posterior means of the MCMCglmm models, as presented in Fig. 2.5. (b).*

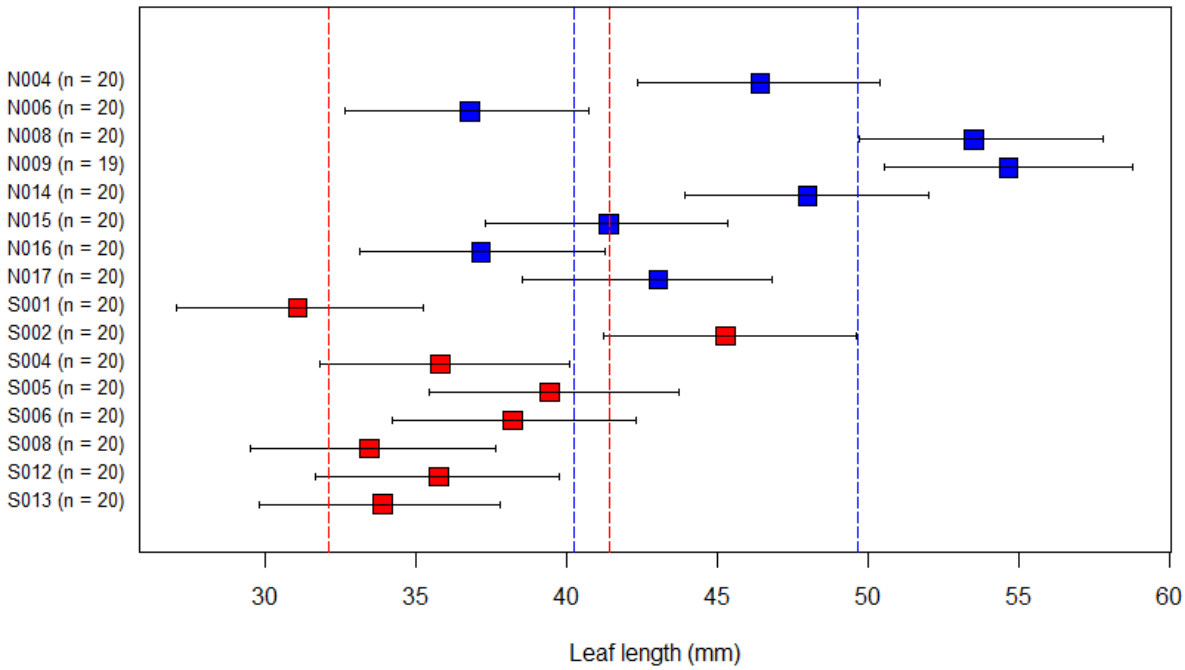

*Figure 2.8 – Posterior means of MCMCglmm models (squares) and 95%*Cis *(horizontal error bars) for leaf length of plants grown from collected from sixteen sites from eight north- (blue) and eight south-facing (red) slope-aspect microclimates after six months of growth. Error bars were generated from HMD interval and represent the 95% credible intervals derived from the MCMC models. Dashed blue and red lines represent the lower and upper 95%*Cis *for the posterior means of the MCMCglmm models, as presented in Fig. 2.5.* l.

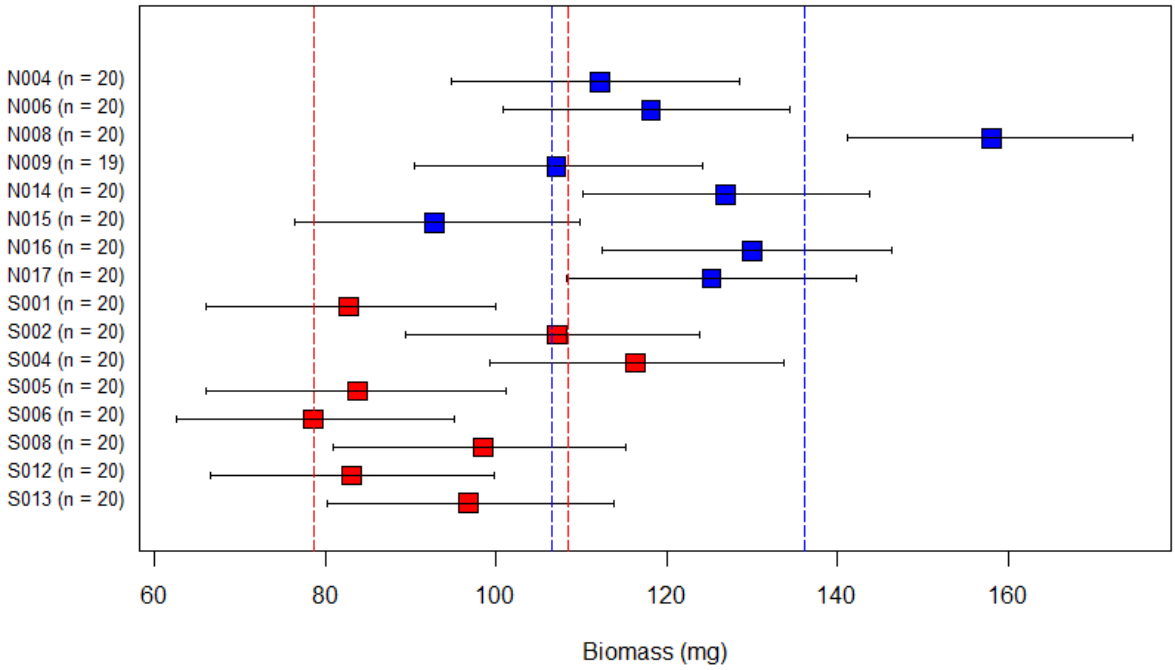

Figure 2.9 – Posterior means of MCMCglmm models (squares) and 95%Cis (horizontal error bars) for biomass collected above 13mm height of plants grown from collected from sixteen sites from eight north- (blue) and eight south-facing (red) slope-aspect microclimates after six months of growth. Error bars were generated from HMD interval and represent the 95% credible intervals derived from the MCMC models. Dashed blue and red lines represent the lower and upper 95%Cis for the posterior means of the MCMCglmm models, as presented in Fig. 2.5. (d).

2.4.2 Leaf tissue traits

Analysis of the leaf tissue traits tissue density and SLA found significant and non/marginally-significant effects respectively for slope aspect of origin (Fig. 2.10). Tissues density was significantly lower for plants from south facing slopes than plants from north facing. SLA was lower for plants from north facing slopes (posterior mean 18.4mm^{2}mg^{-1}, 95%CI 16.9-20.1mm^{2}mg^{-1}) than that of south facing (20.2mm^{2}mg^{-1}, 95%CI 18.6-21.9mm^{2}mg^{-1}), however the result had only marginal statistical support (pMCMC 0.0602).

Figure 2.10 shows that at the population level overlap was seen between the 95%Cis and means for all sites for tissue density, though the means for six of the eight north-facing sites (N004, N006, N008, N009, N014 and N017) fell above the upper 95%CI for south in the by-slope model. The inverse was true for south-facing sites, with six of the eight (S001, S002, S005, S006, S011, S012) having posterior means below the lower 95%CI for north from the by-slope model. Population level analysis of SLA found major overlap between the means

and 95%Cis of all samples with the exception of site S001, with a mean and lower 95%CI greater than the upper 95%CI for either estimate from the by-slope model (Fig. 2.12).

MANOVA analysis found significant effects of slope and site for SLA (slope F = 9.60, *P=0.0021*; site F = 2.14, *P=0.0010*). Tissue density was identified as being strongly affected by slope-aspect microclimate (F = 7.47, *P=0.0066*) but insignificant for site (F = 0.99, *P=0.4597*).

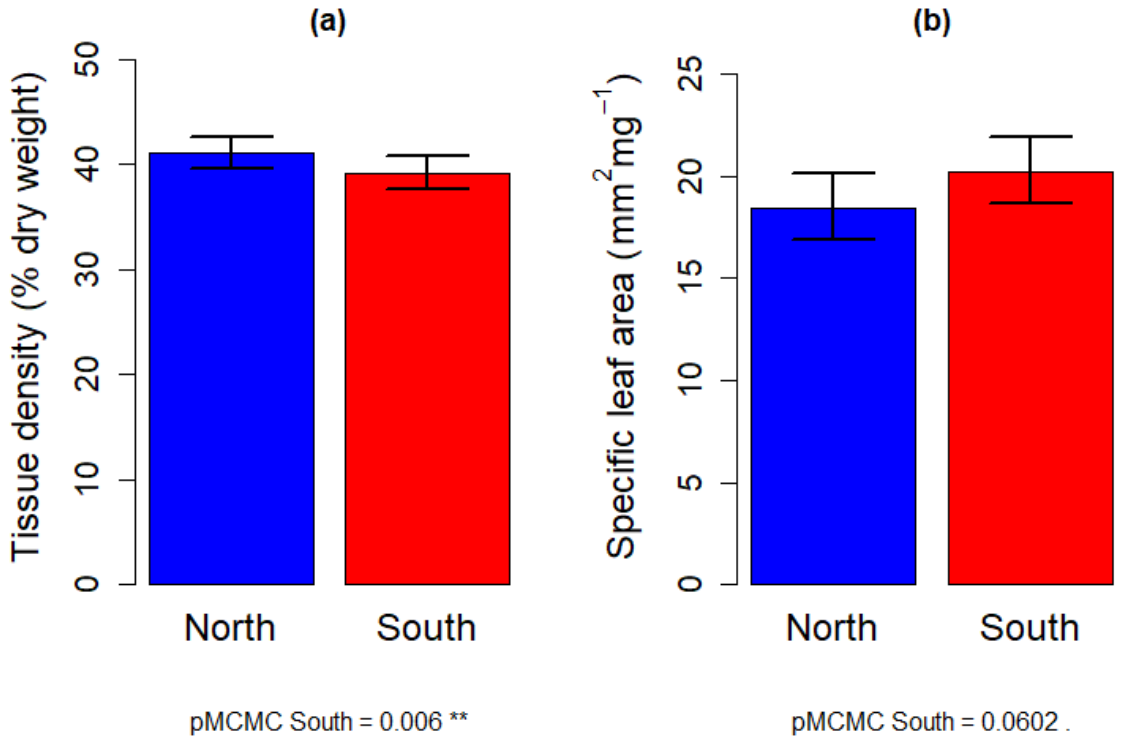

Figure 2.10 – Posterior means of MCMCglmm models for plants grown from collected from north- and south-facing slope-aspect microclimates. Plants were standardised to 10 tillers and a height of 25mm at planting and grown in a randomised fashion in a common garden at Ness Botanic Gardens for 6 months before measurement. Error bars were generated from HMD interval and represent the 95% credible intervals derived from the MCMC models. (a) Tissue density i.e. mass of dry matter as a percentage of turgid mass; (b) specific leaf area i.e. leaf area per unit of dry mass.

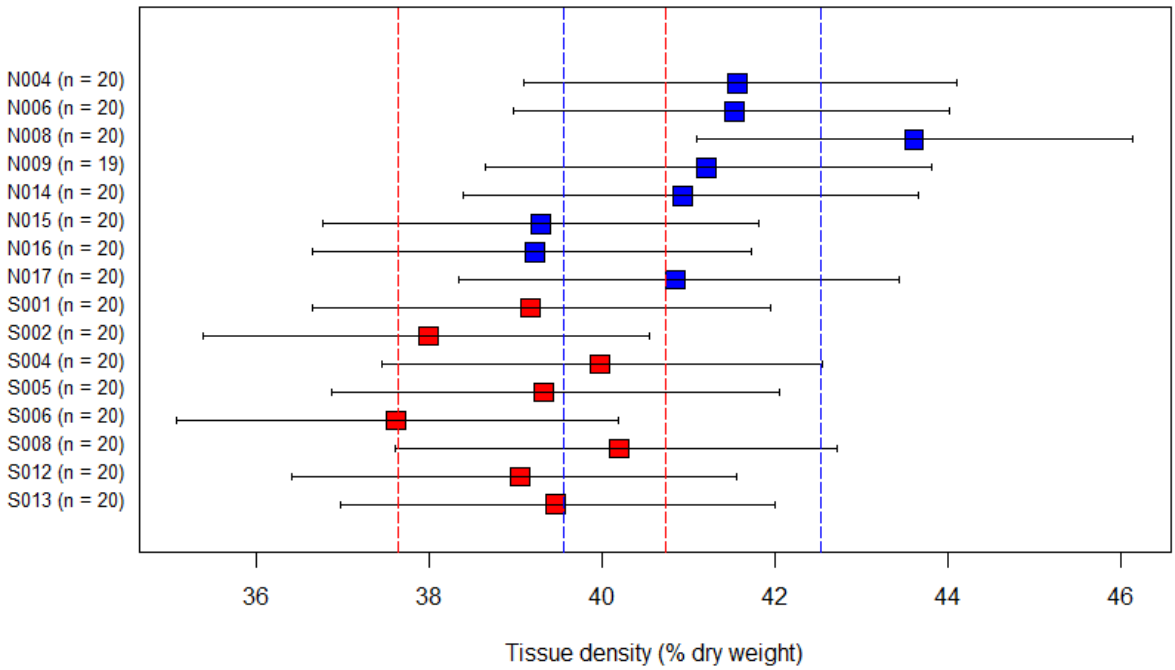

Figure 2.11 – Posterior means of MCMCglmm models (squares) and 95%Cis (horizontal error bars) for tissue density of plants grown from collected from sixteen sites from eight north- (blue) and eight south-facing (red) slope-aspect microclimates after six months of growth. Error bars were generated from HMD interval and represent the 95% credible intervals derived from the MCMC models. Dashed blue and red lines represent the lower and upper 95%Cis for the posterior means of the MCMCglmm models, as presented in Fig. 2.10. (a).

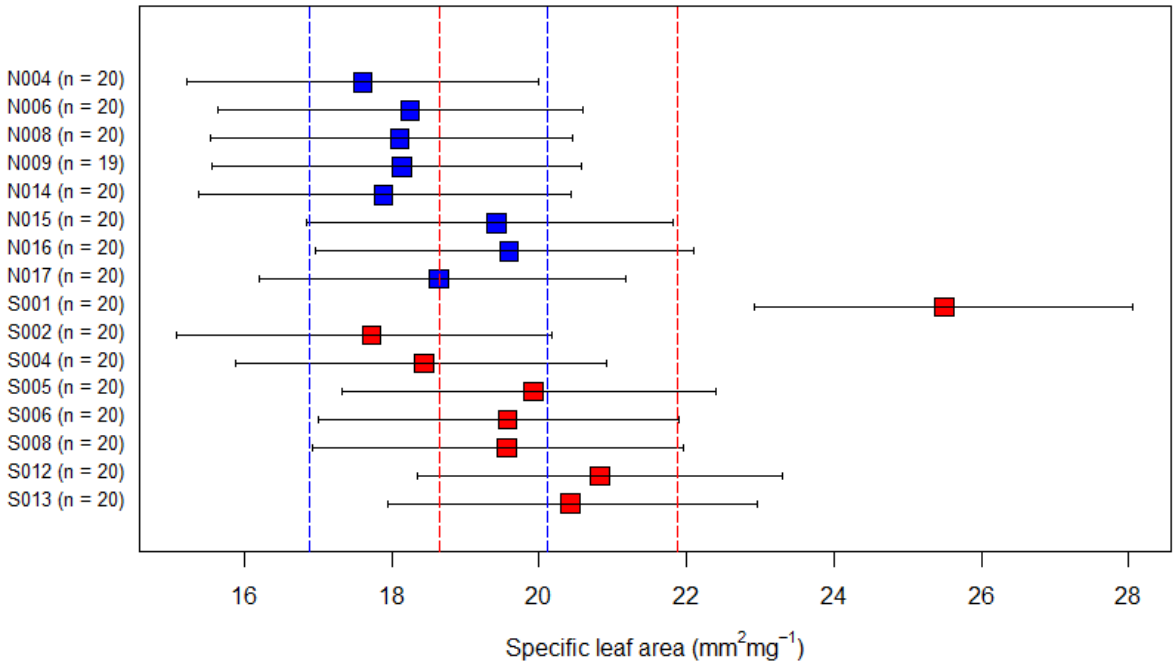

Figure 2.12 – Posterior means of MCMCglmm models (squares) and 95%Cis (horizontal error bars) for SLA of plants grown from collected from sixteen sites from eight north- (blue) and eight south-facing (red) slope-aspect microclimates after six months of growth. Error bars were generated from HMD interval and represent the 95% credible intervals derived from the MCMC models. Dashed blue and red lines represent the lower and upper 95%Cis for the posterior means of the MCMCglmm models, as presented in Fig. 2.10. (b).

2.4.3 Flowering traits

Flowering traits showed a large degree of differentiation in the by-slope models, in all cases, plants from south facing slopes had a greater investment in reproductive effort, producing more flowering tillers overall, more relative to the number of vegetative tillers and with more plants flowering. Plants from south facing slope origins had an average of twice as many flowering tillers than those from north facing slopes (Fig. 2.13 (a)), reflecting a greater proportion of tillers initiating flowering (Fig. 2.13 (b)) as total tiller number was not differentiated for slope aspect.

The proportion of individuals that flowered was also significantly higher for south facing slopes than for north facing, with 63% of south facing plants flowering vs 43% of the north facing plants (Fig. 2.13 (d)). An MCMCglmm model treating flowering probability as a binary categorical trait supported the difference as being statistically relevant (Fig. 2.13 (c)).

MANOVA analysis for flowering traits returned slope and site as significant predictors for all three. In number of tillers and proportion of tillers flowering, slope and site all had p-values <0.0001. Flowering vs. not-flowering had values of 0.0003 and 0.0076 for slope and site respectively.

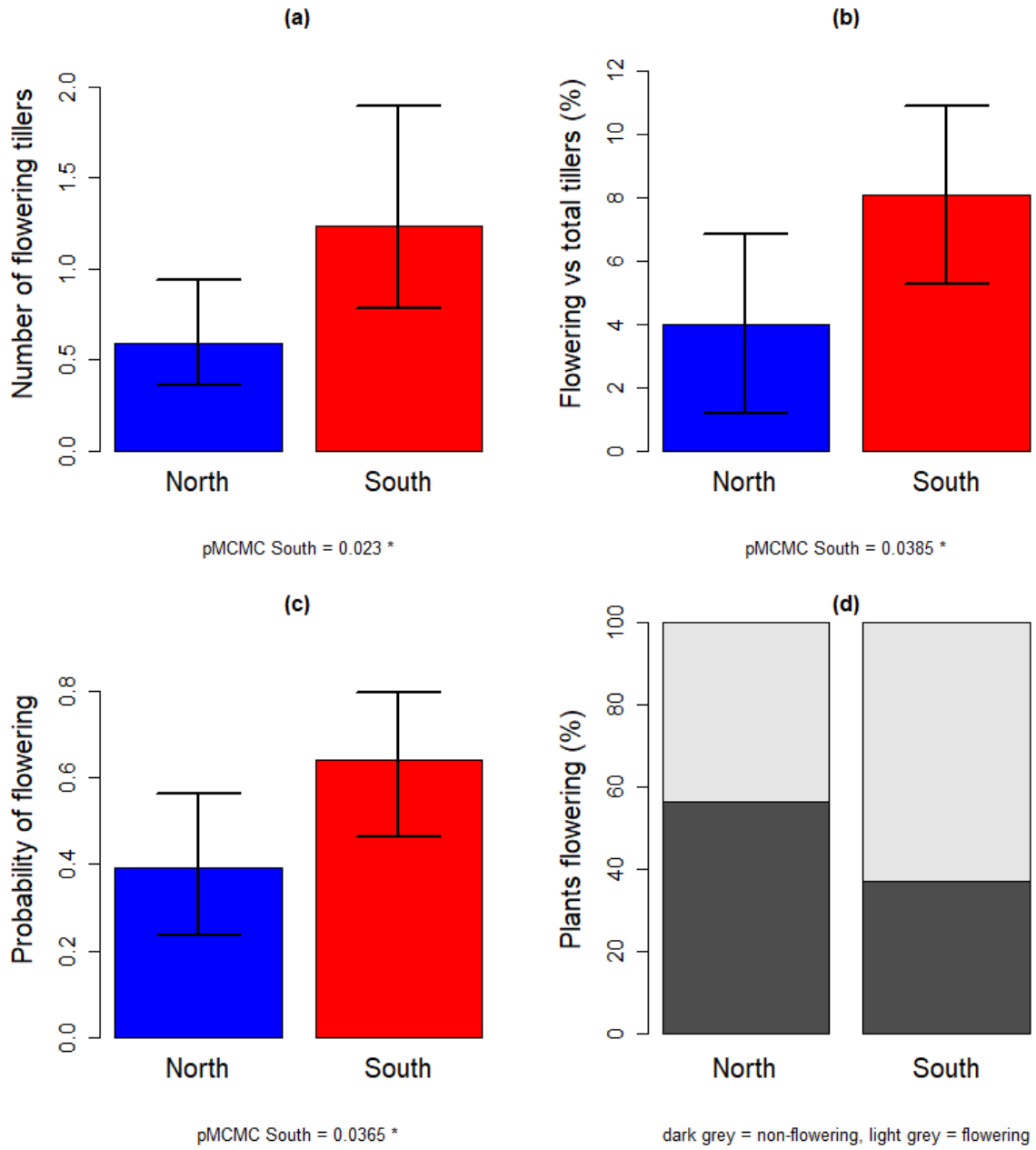

Figure 2.13 – (a, b, c) Posterior means of MCMCglmm models for plants grown from collected from north- and south-facing slope-aspect microclimates. Plants were standardised to 10 tillers and a height of 25mm at planting and grown in a randomised fashion in a common garden at Ness Botanic Gardens for 6 months before measurement. Error bars were generated from HMD interval and represent the 95% credible intervals derived from the MCMC models (a) number of flowering tillers per plant by slope aspect; (b) number of flowering tillers as a percentage of total tillers; (c) probability of plants flowering. (d) Raw data presenting percentage of individuals flowering for different slope aspects of origin.

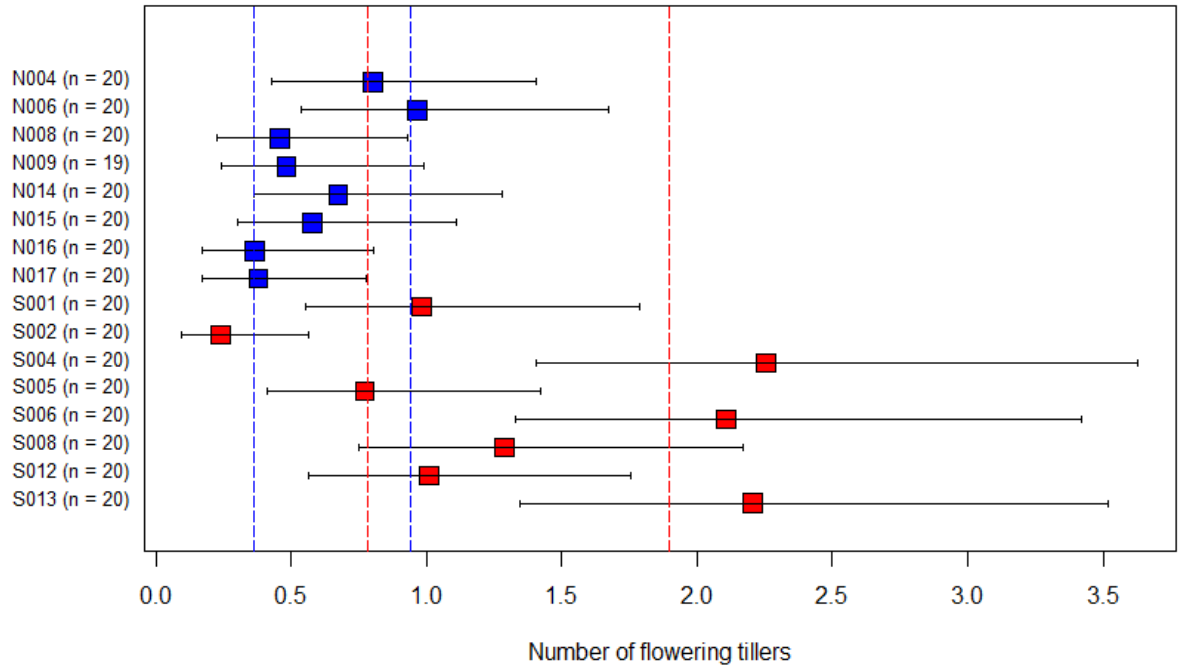

Figure 2.14 – Posterior means of MCMCglmm models (squares) and 95%Cis (horizontal error bars) for number of flowering tillers of plants grown from collected from sixteen sites from eight north- (blue) and eight south-facing (red) slope-aspect microclimates after six months of growth. Error bars were generated from HMD interval and represent the 95% credible intervals derived from the MCMC models. Dashed blue and red lines represent the lower and upper 95%Cis for the posterior means of the MCMCglmm models, as presented in Fig. 2.13. (a).

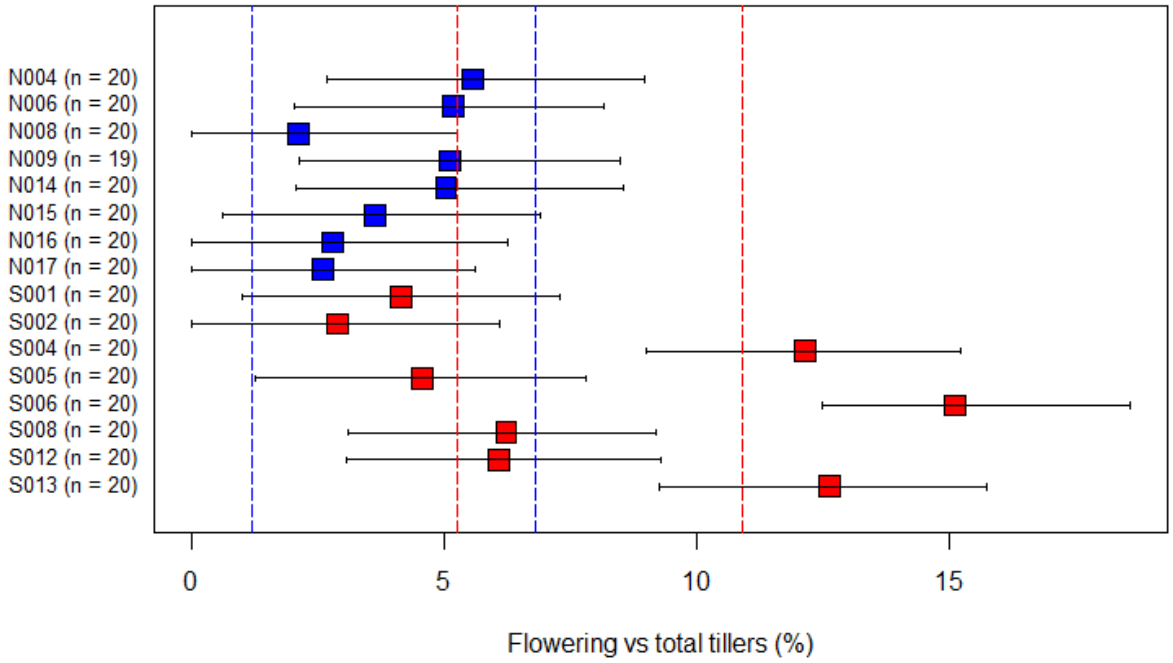

Figure 2.15 – Posterior means of MCMCglmm models (squares) and 95%Cis (horizontal error bars) for tissue density of plants grown from collected from sixteen sites from eight north- (blue) and eight south-facing (red) slope-aspect microclimates after six months of growth. Error bars were generated from HMD interval and represent the 95% credible intervals derived from the MCMC models. Dashed blue and red lines represent the lower and upper 95%Cis for the posterior means of the MCMCglmm models, as presented in Fig. 2.13. (b).

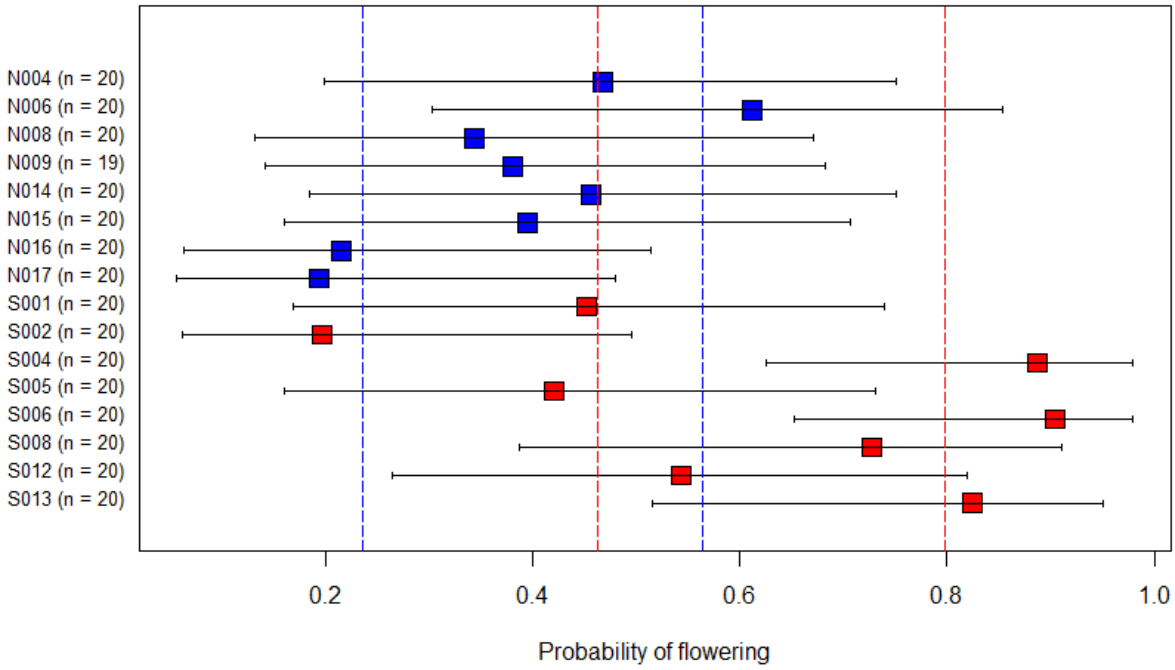

Figure 2.16 – Posterior means of MCMCglmm models (squares) and 95%Cis (horizontal error bars) for probability of flowering of plants grown from collected from sixteen sites from eight north- (blue) and eight south-facing (red) slope-aspect microclimates after six months of growth. Error bars were generated from HMD interval and represent the 95% credible intervals derived from the MCMC models. Dashed blue and red lines represent the lower and upper 95%Cis for the posterior means of the MCMCglmm models, as presented in Fig. 2.13. (c).

2.4.4 PCA

Principal componenta analysis of the measured traits, were plotted agianst each other in Fig. 2.17. No obvious clustering was visible in the plots of PC1 vs. PC2 (a) or PC1 vs PC3 (b), with a lot of overlap between the points representing north and south facing plants. ANOVA analysis of the reultant PCA scores by site and slope (table 2.2) found significant relationships between them and PC1 and for PC2, though for PC3 only slope significantly explained variance, while site did not (F = 1.43, *P=0.1378*). However, the R package PCAtest, a permutational test that compares Phi and Psi values for a PCA to randomly generated matrix, found the resultant PCA to be indistinguishable from random chance.

PC1 explained 34.61% of the variation, and is best explained by variation in height, length and leaf area, all traits concerned with growth habits. PC2's largest explanatory variable was the number of flowering tiller and percentage of tillers that initiated flowering, indicating separation due to reproductive traits. PC3 was explained best by tiller number and biomass,

traits potentially associated with carbon capture (as represented by above ground growth), which may show differences related to environmental stress, particularly water availability.

Table 2.2 – Loadings of traits onto principal components axes and percentage of explained variation, for trait measurements from 20 individuals each of 16 sites

	Comp.1	**Comp.2**	**Comp.3**	**Comp.4**	**Comp.5**	**Comp.6**	**Comp.7**
Tillers	0.247	0.026	0.728	0.064	0.132	0.285	0.501
Height	-0.484	0.127	0.026	-0.030	-0.086	-0.640	0.569
Length	-0.506	0.117	-0.053	0.234	0.125	0.364	0.094
Leaf area	-0.482	0.190	-0.046	0.266	0.141	0.409	-0.057
SLA	0.188	-0.071	0.006	0.684	0.584	-0.369	-0.113
No. flowering tillers	0.220	0.657	0.011	-0.075	0.110	0.037	0.124
Biomass	-0.287	0.182	0.652	-0.053	-0.114	-0.255	-0.611
Tissue density	-0.163	-0.136	0.009	-0.622	0.752	-0.008	-0.039
% flowering tillers	0.157	0.668	-0.197	-0.079	0.087	-0.077	-0.104
% of variation	*34.61*	*20.68*	*14.75*	*13.80*	*7.87*	*4.49*	*2.32*

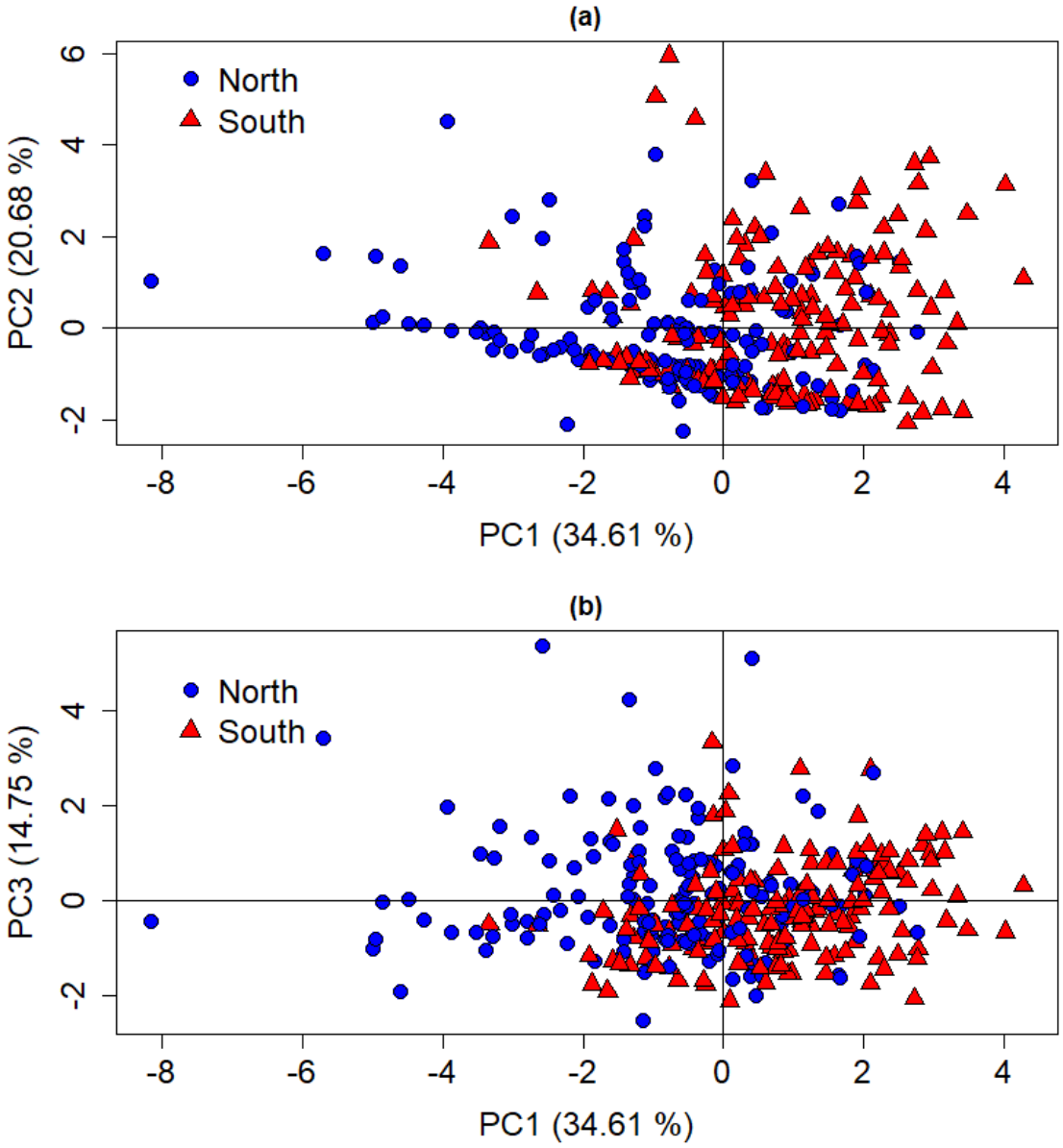

Figure 2.17 – PCA plots of all individuals using the traits tiller number, plant height, leaf length, leaf area, SLA, number of flowering tillers, biomass, tissue density and percentage of tillers flowering. Points marked for slope-aspect of origin according to legend. PC1 plotted against (a) PC2 and (b) PC3

2.4.5 LDA

A linear discrimination analysis was performed on the traits that showed appropriately normal distribution: tiller count, plant height, leaf length, leaf area, SLA, biomass, tissue density and percentage of tillers flowering. The output of the test showed some separation but appreciable overlap between the populations. Coefficients of the LDA are reported in table 2.3, identifying leaf area as the largest component of discrimination. The largest

influencing value reported was leaf length, at -0.1134, followed by leaf area at 0.075, indicating mild importance of leaf morphology in distinguishing slope aspects.

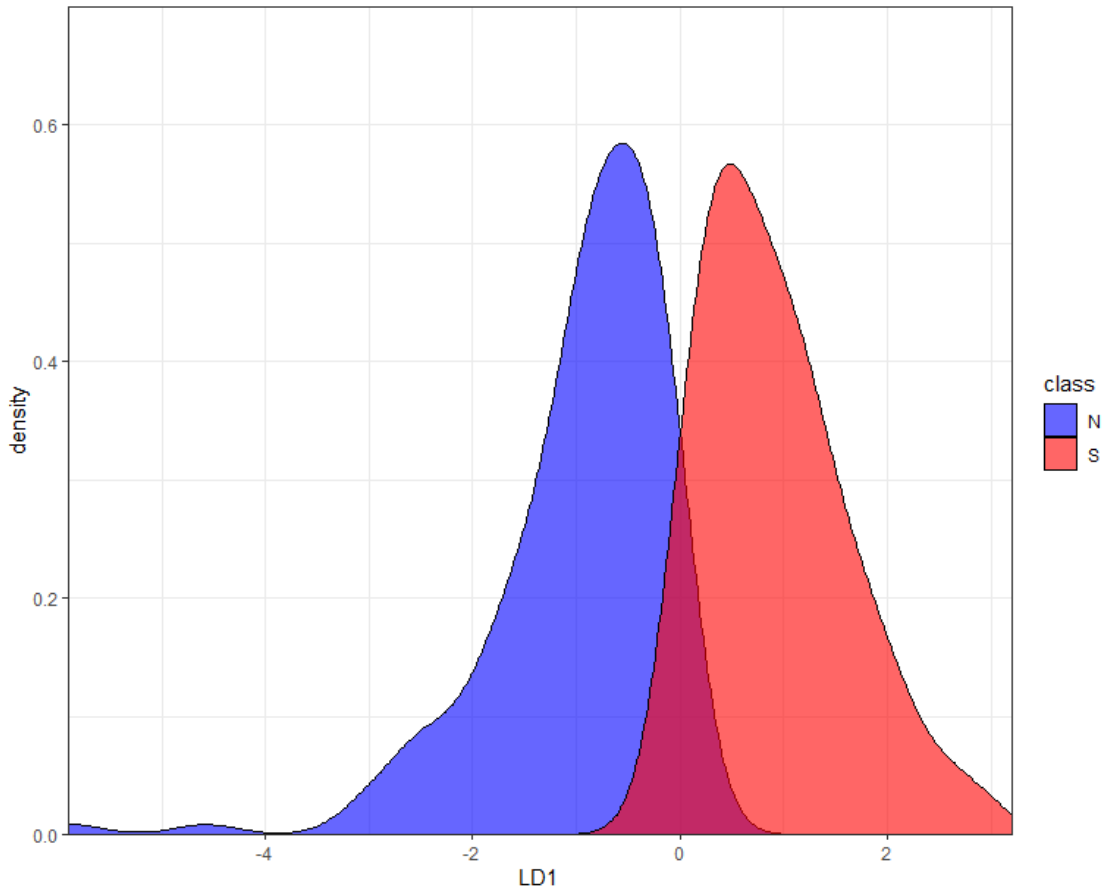

Figure 2.18 – Density plot of a linear discrimination analysis performed using traits tiller count, plant height, leaf length, leaf area, SLA, biomass, tissue density and percentage of tillers flowering.

Table 2.3 – Coefficients of linear discrimination as reported by the LDA model

Trait	**LD1**
Tillers	0.019664497
Height	-0.029957974
Leaf length	-0.113419817
Leaf area	0.075254697
SLA	0.004582332
Biomass	-0.013703047
Tissue density	-0.010084100
% of tillers flowering	0.046358695

2.5 Discussion

The aim of this study was to investigate whether microclimatic selective pressures were driving evolutionary differentiation in plant phenotype between north and south facing populations. I found evidence suggesting moderate differentiation between populations based on slope aspect microclimate and on the level of individual populations through morphological analysis supported by Bayesian generalised linear mixed models and multivariate analysis of variance. These results suggest that, on average, *F. ovina* populations on north-facing slopes are taller than those from south-facing slopes, with longer, denser leaves, and significantly more biomass above 13mm in height. Plants from south-facing slopes are characterised by greater numbers of flowering tiller (both absolute and relative to vegetative tillers) and a higher incidence of flowering than those from north-facing slopes. PCA and LDA both supported the findings of overall trends differentiating north and south facing slope populations, but with a great deal of overlap too. Both analyses identified leaf length as particularly significant sources of variation across all traits, with MANOVA and MCMCglmm finding slope-aspect to be a significant predictor of leaf length amongst all growth traits and individually (respectively). However, between-site variation in these traits was high, and no specific pattern could be said to generalise neatly to all north or south facing slopes – as evidenced by the conflicting results on the significance of the traits from multivariate analyses.

The major differences seeming to differentiate slope-aspect microclimate populations from each other are in growth-habit and reproductive strategy. Plants from north facing slopes typically had longer leaves and greater overall height, a trait evident in the field and retained in the common garden. The adaptive value of these traits is seemingly straightforward: north facing slopes visited in this study generally had a more obvious deep bryophyte layer and shorter sward – in order to reach the light, plants must be taller. However, whether this is genetically fixed, rather than simple etiolation of plants under conditions of light competition, may have important implications for restoration work. While this trait is associated with slope aspect, it may be a response to co-occurring vegetation rather than the direct effects of climate as site S002 also displayed this phenotype. Though a population from a south-facing slope, the site itself is somewhat overgrown, with a noticeable bryophyte and shrub layer (extensive and deep cover of

Helianthemum nummularium), raising the possibility that biotic effects associated with microclimate drive selection for this phenotype.

Tissue density showed a very strong association with slope aspect: plants from south-facing slopes had a significantly lower tissue density. This may be explained as a response to the increased drought stress south facing slopes are typically exposed to, relative to north-facing slopes (Bennie *et al.*, 2006; Kimball *et al.*, 2017; Rorison *et al.*, 1986). Lower tissue density has been associated with drought tolerance/avoidance in many plant species, most obviously in the form of succulence, but also in various non-succulent tree species (Ryser & Aeschlimann, 1999; Wolfe, 2017). Succulence however is exceedingly rare in grasses (Ho *et al.*, 2019), so a more likely explanation is disposability of "cheaply built" leaves is selected for under conditions of regular drought-associated loss of leaves. Regardless of the specific mechanisms at work, plastic and fixed responses between populations for the tissue density of lamina are known as responses to both light and water availability in grasses. Contrary to my findings in *F. ovina*, the grass *Sesleria nitida* studied on north vs south facing slopes, on limestone, in a valley in alpine northern Italy showed an inverse relationship, with tissue density being lower for north-facing slopes than south (Wellstein *et al.*, 2013).

Aboveground biomass accumulation above 13mm was significantly higher for north facing plants. As belowground biomass was not measured, it's possible that total biomass was equal but plants from south facing slopes were apportioning relatively more biomass to tissues below the 13mm height cut-off. Differential allocation of resources resulting in higher root:shoot ratios have been found associated with both plastic and fixed responses to drought in grasses such as *Agrostis capillaris* (Rapson & Wilson, 1992). Similar responses are known for the grass *Poa nemoralis* where reduced light availability increases the allocation of biomass to aboveground tissues (Plue *et al.*, 2020). The difference in biomass may also be explained as a response to relative changes in light availability for north vs. south facing plants occurring in the common garden: Insolation rates differ between slopes and flat ground, with north facing slopes receiving less radiation, and south facing slopes receiving more, due to angle of incidence of sunlight on the ground (Rorison *et al.*, 1986). As the experimental common garden was on a flat level, south and north facing plants were therefore receiving, respectively, less and more sunlight than they had. Comparing between different grasses, low-light adapted species such as *Deschampsia flexuosa* show faster

increases in biomass accumulation with increased light than shade intolerant species such as *F. ovina* even past the compensation point for both species (Mahmoud & Grime, 1974). A further potential explanation for the strength of the observed difference is that, due to the shorter average height of plants from south-facing slopes, more biomass escaped harvesting.

Reproductive traits showed some of the clearest responses to slope-aspect microclimate, as measured by number of flowering tillers, % of tillers flowering and number of flowering plants showed a clear difference by slope aspect of origin. Plants from south facing slopes were more likely to flower (63% of south plants flowered vs. 44% of norths), and had both higher absolute (2.64 vs 1.88) and relative (8.35% vs 4.24%) investment in flowering tillers. These results are comparable with studies on several grasses, in which increased rates of sexual reproduction is associated with more open and sunny sites vs. shaded ones (Kottler & Gedan, 2022; Plue *et al.*, 2020; Rapson & Wilson, 1992). The grass *Poa alpina* has highly variable levels of reproductive investment at the population level with little climatic influence on actual chance of flowering (Hamann *et al.*, 2016). Continuous selection for increased drought at the BCCIL has been shown to influence reproductive traits in *F. ovina*, with plants descended from drought plot parents flowering earlier than those from control plots. However, contrasting with my findings on tiller numbers for north vs south facing plants, drought plot ancestry at the BCCIL resulted in fewer flowering tillers relative to controls (Trinder *et al.*, 2020).

Given the context, these findings together suggest that microclimate may be driving selection for differing life-history strategies in populations of *F. ovina* on north and south facing slopes. All *F. ovina* are stress-tolerators, the C-S-R strategies exist as a continuum. Viewed through the lens of universal adaptive strategy theory (Grime, 1977) it can be thought of as pushing south facing slope populations towards a more ruderal-like strategy – grow cheap and reproduce fast. Simultaneously, north facing populations are pushed more towards stress-tolerator strategy – weathering the resource scarcity with investment in vegetative growth and durable leaves. Comparison might be drawn with species of the (polyphyletic) *Festuca* subgenus *Vulpia*; species which have transitioned from the ancestral perennial state to an annual or ephemeral lifestyle in habitats characterised by extreme, annual droughts (Catalán *et al.*, 2004; Díaz-Pérez *et al.*, 2014; Torrecilla *et al.*, 2002).

Site-by-site comparison revealed significant variation between all sites for most traits, indicating there are other factors than slope aspect influencing phenotype. As this study did not measure soil depth, nor have data on site management history, these cannot be ruled out as possible factors. Soil depth heterogeneity at the BCCIL has been demonstrated to influence trait differentiation between the responses of populations of *F. ovina* and *Plantago lanceolata* to climate manipulation (Ravenscroft *et al.*, 2014), making it a possible source of site-to-site variation in response to microclimate.

Management history and practice will also vary by site, with differing grazing regimes known to affect leaf tissue traits such as tissue density and SLA. A study on four pasture management systems in Italy found that these traits respond strongly to grazing intensity (Targetti *et al.*, 2013). Defoliation in the form of mowing can drive plastic and evolutionary responses in the growth habit of grasses, phenotypes of *A. capillaris* from mown samples had a lower growing phenotype with less Investment in leaf tissue (Rapson & Wilson, 1992). Different sites have different grazing regimes on them differing in intensity, seasonal timing and type of livestock (sheep/cattle/both). When allowed free range, sheep are known to have preferences associated with slope aspect (Plaza *et al.*, 2022). In Spain, sheep show a distinct preference for grazing north facing slopes, likely as escape behaviour from heat and, due to the arid climate of much of Spain, increased water availability leading to better grazing. Whether or not this, or even the reverse, is seen in the cooler, wetter climate of Britain, grazing site preference in sheep has the potential to exert selection pressures on CG2 plants like *F. ovina*. However, most sites in the Peak District are enclosed grazing, meaning grazing based selection pressure is likely influenced more by land management than slope-aspect preference in sheep.

The high levels of site-by-site variation found in this study may be an effect of the naturally variable morphology of *F. ovina* (Harberd, 1962; Stace *et al.*, 1992; Watson, 1958), but may be a feature of the selected site. Only eight sites were selected for each slope aspect, and relatively few morphological characteristics measured. Future avenues of study might use a greater number of replicates either in sites or in number of individual plants, a greater length of time in a common garden or heritability studies using collected seed and half-sib families. None the less, the data presented here provide credible evidence for general

morphological trends between slope aspect, evidencing consistent patterns of evolutionary differentiation by slope-aspect microclimate.

With regard to the possibility that the observed differences are a result of retention of plastic differences in response to collection site, Watson's 1958 paper noted that morphology of *F. ovina* was subject to change once placed under uniform culture conditions. In that paper, plants that in the field were identified as *F. tenuifolia*, assumed a growth form more typical of *F. ovina* in the common garden. The work of Dr Sarah Trinder on a *F. ovina* F1 progeny array derived from plants from the control and drought treatments at the BCCIL[11] also provides support for the use of standardised clones in phenotypic analysis. Chapter 5 of her PhD thesis found significant phenotypic difference between clones grown for several years under standard conditions both in relation to environmental ancestry and in differential response of clones of the same plants to a two-month simulated drought (Trinder, 2017). Further to that, while not recorded here, I can attest that most of the plants from S001 were at time of collection, below the 25mm standardisation height, yet upon measurement at the end of the experiment had a mean height of 35.5mm.

To conclude the evidence presented here, north and south facing slope aspect populations show significant variation between sites, however statistical support is strong for the overall effect of slope on the level and direction of variation. The retention of these field-observed trait differences in the common garden indicates a level of genetic control may be directing them. The nature of the traits can be related back to environmental differences between slope aspects, with water and light availability likely linked to trait differences observed in growth form and reproductive investment, with higher risk of drought stress/drought events favouring a phenotype with reduced above-ground biomass accumulation, less investment in leaf tissue and greater investment in sexual reproduction; while the phenotypes observed in north-facing slope-aspect populations tends towards traits associated with longer term retention of leaf tissue, light capture under limitation, and vegetative competition rather than sexual reproduction.

[11] For more details on the BCCIL and the relevant treatments, see section 4.3.1 of this thesis

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3 The influence of topological microclimatic variation on the genetic structure of the grass *F. ovina*

3.1 Abstract

Plant adaptation to environmental conditions is underpinned by heritable changes that provide a fitness advantage. The phenotypic and genetic shifts that accompany adaptation are known to occur at the sub-metre scale and in timescales under 20 years when selection pressures are sufficiently high. It therefore follows that climatic and microclimatic variation in the landscape should drive similar adaptive responses to selection. However, although adaptive responses to the climate have been detected at biogeographical scales in plants and across large altitudinal gradients, little is known about the impacts of fine-scale microclimatic variation at finer scales in natural landscapes. In order to understand how fine-scale variation in microclimatic conditions shape genetic structure in the landscape, I used a system of sites on north and south facing valley slopes in the Peak District as a natural source of microclimatic variation across a landscape. North facing slopes (n = 8) represented cooler, damper conditions, while those that were south-facing (n = 10) were warmer and drier. Genotyping-by-sequencing (GBS) was applied to populations of the grass *F. ovina* from these sites, using a pooled sample strategy, where individuals from the same site (population) were combined into a single sample. Returned sequence reads were aligned to the *F. ovina* reference genome and > 15000 SNP variants were called following standardised methods for GBS data. Genome-wide genetic differentiation between *F. ovina* populations was generally low $F_{ST} < 0.1$, as expected for a wind-pollinated perennial plant that typically maintains large population sizes. However, four of the eighteen populations were significantly genetically differentiated from all remaining populations and from each other, indicating an unexpectedly low level of either gene-flow or effective population size in these sites. I observed a low level of background differentiation between slope aspect microclimates (median $F_{ST} = 0.004$). However, I also identified seventeen loci with a signature of ecological adaptive genetic differentiation linked with slope aspect microclimate. The microclimatic conditions of north- and south-facing slope aspects in the Peak District appear to be selecting for genes with functions related to drought tolerance, cold tolerance, pathogen defence and reproductive phenology. I conclude (i) that fine-scale

microclimatic variation drives ecologically adaptive genetic change at an equally fine spatial scale, (ii) that the selective pressures driving this adaptation are potentially linked to abiotic conditions or microclimate-associated shifts in the biotic environment and (iii) that fine-scale microclimatic variation allows adaptive genetic variation to be stored in landscapes, providing a potential buffer against the effects of anthropogenic climate change. The results presented here agree with the findings of the previous chapter, supporting the role of slope-aspect microclimate and individual sites as sources of important adaptive genetic variation in *F. ovina*.

3.2 Introduction

Climate change is already affecting plant population structure, survival and distribution (Ackerly, 2003; Angert *et al.*, 2011; Jones & Gilbert, 2016; Román-Palacios & Wiens, 2020) and is expected to increase in magnitude and effect in the coming century (Cunze *et al.*, 2013; IPCC, 2014). Predicted changes in climatic conditions over the coming century however may exceed the adaptive potential of many plant populations, putting them, and the other species that depend upon them, under threat of extinction (Niskanen *et al.*, 2019; Román-Palacios & Wiens, 2020; Sales *et al.*, 2020; Suggitt *et al.*, 2018). Genetic diversity within populations is key to adaptive potential, with differential selective pressures driving its evolution and maintenance at specific loci. Heterogeneous environments consisting of diverse microclimatic patches, applying different selective pressures, are expected to foster higher levels of adaptive variation (Denney *et al.*, 2020). This presents a challenge for ecologists and conservationists alike as we try to forecast the effects climate will have on vulnerable ecosystems and design mitigation strategies (Angert *et al.*, 2011; Neale, 2012). One focus in this is the identification of sources of "pre-adapted" genetic variation in naturally occurring plant communities (Aitken & Bemmels, 2016; De Kort *et al.*, 2020), as, at an ecosystem level, both species level and within-species genetic diversity buffers populations and communities against the deleterious effects of climate change (Fridley *et al.*, 2007; Grime *et al.*, 2000; Norberg *et al.*, 2012; Vellend & Geber, 2005).

Adaptive genetic differentiation between populations is driven by selection for local biotic and abiotic conditions. In order for populations that are not reproductively isolated from

each other to become genetically differentiated, selection pressures must first overcome genetic drift (Masel, 2011). Conversely, gene flow between populations and common selective pressures can maintain genetic homogeneity. Some of the earlier studies on fine-scale population differentiation come from studies on the effects of edaphic conditions, such as evolution of lead tolerance in *Agrostis tenuis* populations at Trelogan in North Wales (Antonovics & Bradshaw, 1970) and the Park Grass experiment at Rothamsted. The Park Grass experiment is a long-term soil-manipulation experiment located at the Rothamsted in the England, started in 1856 with fertiliser application, and liming treatments from 1903 onwards. Plots are of variable size but average 17x36m, with sharply defined borders between them (Snaydon & Davies, 1972). Local scale adaptation has been observed between *Anthoxanthum odoratum* populations from different plots in the Park Grass experiment, which showed differences in height, growth habit, growth rate, seasonal patterns of growth, reproductive strategy and disease resistance in response to 71 years soil amendment (Snaydon & Davies, 1972).

Climate, and microclimate, are strong selective drivers of evolutionary adaptation in plants. Microclimate, in the form of number of frost days and mean temperature, was shown to be a powerful predictor of frost hardiness in alpine *Arabidopsis thaliana* populations (Lampei *et al.*, 2019). Martins *et al.* identified loci under selection related to climatic differences in 103 *Quercus rugosa* specimens collected from across Mexico using genotyping-by-sequencing to identify 97 SNPs associated with climatic variation (Martins *et al.*, 2018). A study investigating divergence between a duplicated *hsp17* gene in *Hordeum spontaneum* at Evolution Canyon I, Israel, discovered several hundred SNPs in the two copies, with signs of differentiation between those from different microclimate populations suggesting adaptive benefit (T. Zhang *et al.*, 2014). Two studies at the Buxton Climate Change Impacts Laboratory (BCCIL) demonstrated strong phenotypic evidence of evolutionary change in *Plantago lanceolata* (Ravenscroft *et al.*, 2014) and adaptive genetic changes in both *F. ovina* and *P. lanceolata* (Ravenscroft *et al.*, 2015), in response to fifteen years of climate manipulation for winter temperature and water availability. A study on the effects of climate manipulation on a population of the Mediterranean shrub *Fumana thymifolia* found that early acting effects, such as differential seedling establishment in this case, can serve as powerful drivers of rapid evolutionary response to climate (Jump *et al.*, 2008). A review by

Denney *et al.* concluded that, in heterogenous habitats, genotypes adaptive for different climatic conditions are often fostered by microclimatic variation, with potentially beneficial effects in population level response to climate change (Denney *et al.*, 2020). Together these studies demonstrate the potential for plant populations to show adaptive and genetic differences associated with climate differences at large and small scales.

This knowledge has practical application in conservation and restoration ecology, in the restoration of degraded habitats and the creation of new habitat patches (Kapás *et al.*, 2020; Morris *et al.*, 2006; Pywell *et al.*, 2011; Schröder, 2008). A common practice is the introduction or reintroduction of genotypes or species to a habitat, with the choice of propagule source referred to as provenancing. Climate aware strategies for which can take the form of both composite and predictive provenancing (Aitken & Bemmels, 2016; Breed *et al.*, 2013; Prober *et al.*, 2015). Briefly, predictive provenancing is the process of identifying suitably adapted propagule sources based on forecasted conditions at the planting site. Composite provenancing is strategy in which new populations are created by combining propagules from a number of populations, casting the genetic net wide and allowing natural selection to generate a robust, well adapted population. Both have their merits and both have the potential to benefit from greater understanding of local and distant sources of adaptive genetic variation.

In this chapter I aim to address the question of whether microclimatic selection pressures drive evolutionary differentiation in plant genotype, using natural populations of the grass *F. ovina* in a well-replicated, spatial mosaic of north and south facing topographical slope aspects and their associated contrasting climatic conditions as a study system. The work presented in chapter 2 of this thesis provides evidence that populations on north and south facing slope aspect microclimates in the Peak District show phenotypic differentiation between populations, but provides no information on the genetic changes that may underpin this differentiation. Furthermore, population genetic analysis will allow for a measure of understanding of the levels of gene flow vs selection between sites, of particular interest due to high levels of site-by-site variation in many traits in the previous chapter. To investigate this, I used genotyping-by-sequencing to identify single-nucleotide-polymorphisms in pools of ninety-four plants each from twenty-two sites (ten north facing, twelve south facing) within a 26x11km area of the Peak District. By comparing the resultant

allele frequencies obtained from these analyses I was able to identify loci under selection, for slope aspect. Though all slopes differ, using replicated north and south facing slopes within a relatively small area, where bedrock chemistry and the overarching climate remain constant, allows for the identification of observed differences as explained by slope-aspect microclimate.

I show that populations of *F. ovina* plants from north and south facing slopes in the Peak District mostly have low levels of differentiation from each other, but feature a number of loci with clear patterns of selection between populations related to slope aspect. Climatic differences between north and south facing slope aspects are selecting for differentiation in loci related to disease resistance, annual phenology, tolerance of abiotic stress and nutrition acquisition. However, though gene flow seems to be regular between most populations, as distinct subset of four sites show far greater levels of divergence that cannot be explained by slope aspect.

3.3 Methods

3.3.1 Site selection

Sampling sites consisted of CG2 grasslands (Rodwell, 1992) oriented facing within 10° of magnetic north or south. Potential sites were identified from known sites recommended by Dr Raj Whitlock, as well as areas with appropriate slope aspect from Ordinance Survey maps of the area. Sites were then visited in person and inspected for plant community and size, to ensure they were suitable, where possible, pairs of adjacent and opposites sites were included, however in many cases only the north or south facing slope of a dale was acceptable. Twenty-two of the selected sites were considered appropriate sampling locations for study, consisting of 10 north facing slopes and 12 south facing (table 3.1).

Table 3.1 – site codes, names and locations using 10 figure Ordnance Survey grid references, for map, see figure 1.1.

Site code	Site name	Grid reference	Aspect
N003	Longdale N	SK 13935 61888	N
N004	Gratton Dale N	SK 20081 59680	N
N006	Hay Dale N	SK 12315 76480	N
N008	Chee Dale N	SK 11341 72504	N
N009	Topley Pike	SK 10031 71868	N
N013	Coldeaton	SK 14652 56057	N
N014	Deep Dale, Sheldon	SK 16756 70113	N
N015	Priestcliffe Lees	SK 15616 72949	N
N016	Cressbrookdale N	SK 17410 74548	N
N017	Deep Dale, Sheldon -2	SK 16142 69494	N
S001	Mill Dale	SK 14244 54992	S
S002	Hall Dale	SK 13537 53634	S
S003	Longdale S	SK 14128 62237	S
S004	Gratton Dale S	SK 20057 59746	S
S005	Hay Dale S	SK 12385 76537	S
S006	Mon''s Dale	SK 13753 73686	S
S007	Chee Dale S	SK 11351 72792	S
S008	Wye Dale	SK 09887 72514	S
S009	Lathkill Dale	SK 17529 65542	S
S011	Tansley Dale	SK 16999 74727	S
S012	Cressbrookdale S	SK 17305 75391	S
S013	Stepping Stones	SK 15120 51516	S

3.3.2 Sample collection

Collection of samples was made using a 9x9m quadrat, to create a grid of 10x10 points (for a total of 100 samples), spaced 1m apart. While *F. ovina* is a weakly spreading plant, its growth habit generally described as tussock forming (Stace *et al.*, 1992), over time clones may spread and ramets *possibly* may be found at a distance of 8 metres from each other (Harberd, 1962). However, 1 metre apart was decided upon as a sensible distance to avoid resampling the same plant unless an individual genotype makes up a significant portion of the population, in which case its addition to the pool multiple times would be representative of the population.

Samples were collected in the form of 2-5 tillers, dependent on leaf length, with the aim of harvesting approximately 20-40mg of dry material. Tillers were stripped of dead leaves and

leaf-sheaths before placing in silica gel, an established method for preserving/preparing plant material for DNA extraction (Chase & Hills, 1991). Samples collected in drier weather were placed directly into labelled, screw-top O-ring tubes, filled ¾ with self-indicating silica gel in the field. Samples collected in wet weather were placed into labelled Eppendorf 1.5ml microfuge tubes with 10µl of deionised water in the field, and blotted and transferred to the tubes of silica gel in the evening of the same day.

For several weeks after collection, tubes were visually inspected for saturation of the silica gel (indicated by colour change from orange to white/clear), however none required replacement.

3.3.3 DNA extraction

DNA was extracted from 94 of the 100 samples collected from each site in a 96 well format, silica binding protocol, using a CTAB based extraction buffer and guanidium hydrochloride based binding buffer (Werth *et al.*, 2016).

7-15mg of silica-dried *F. ovina* material from each sample was placed into a 1.2ml collection microtube (Qiagen), with a 3mm tungsten-carbide bead (Qiagen), capped and ground in a Tissuelyser II (Qiagen) at 25Hz. Due to the tough nature of *F. ovina*, the most effective way to ensure all 188 samples were sufficiently homogenised, samples were run for two runs of 15 minutes.

Once homogenised, 450µl of extraction buffer (2% w/v CTAB, 1.4M NaCl, 20mM EDTA, 100mM Tris pH 8, 2% w/v PVP), 1µl RNAse A (20 mg ml^{-1} NEB Monarch) and 1U Proteinase K (NEB) were added to each tube. The tubes were capped and mixed by manual shaking until all material was dispersed in the buffer, then spun briefly at 300G for 30 seconds to keep foam away from lids. Racks were placed in a water bath at 65°C to incubate for 15', with gentle inversion to mix at 5' and 10'.

After incubation, tubes were spun at 4400G for 25' in a plate centrifuge (STARlabs) to separate the extraction buffer from cell debris. 400µl of supernatant was pipetted off from each tube to a separate well in a clean, 1.2ml (Thermo-Fisher) 96 well storage plate, along

with 600µl of GuHCl binding buffer (2M guanidium hydrochloride, 33% v/v 1xTE, 67% v/v EtOH[12]), which were mixed by gentle pipetting.

Each sample was then transferred to a separate well in a 96-well, 1.2ml glass-fibre filter plate (PAL) set above a vacuum manifold. The solution was drawn through the filters with the vacuum manifold set as low as possible to allow for greater contact time between the chaotropic-salt-DNA solution with the silica in the filter, until all liquid was gone. The filter membranes were then washed with 500µl of 75% EtOH, by drawing it through the membrane, and the wash step repeated a second time.

Following washing, the plates were spun at 4400G for 1' to remove excess wash buffer from the membrane, and allowed to air-dry on the bench top for 10'. At the end of the drying time, DNA was eluted directly into a 96-well rimless PCR plate (STARlabs). Elution was done by adding 100µl of 80°C low TE buffer to each well of the filter plate and allowing it to incubate on the bench for 5 minutes, followed by spinning in the plate centrifuge at 1000G for 1' followed by 4400G for 1'. Plates were then labelled and sealed with PCR membrane (Thermo-Fisher), and stored at -20°C.

3.3.4 Library preparation

DNA was quantified using the Quant-iT HS dsDNA assay from Thermo-Fisher in a 384 well format. Greiner bio-one, black polypropylene plates were used to perform the assay in a reduced volume of 20µl and a BioTek plate reader. Once quantified, DNA was pooled by site of origin in an equimolar fashion, and cleaned using the AMPure XL system (Beckman) and eluted into nuclease-free water. The cleaned pools re-quantified using QuBit HS dsDNA assay (Thermo-Fisher) and normalised to 20ng μl^{-1}.

Cleaned pools were double-digested using a pair of the methylations sensitive restriction enzymes and ligated to barcoded adaptors in a combined restriction-ligation step. Fragments were generated by using EcoRI-HF (NEB) as a rare-cutter (RC) and HhaI-HF (NEB) as a common-cutter (CC), and ligated to barcoded adaptors with T4 ligase (NEB), using an alternating cycle of 37°C – 1' (restriction) followed by 25°C – 1' (ligation) 100 times, followed

[12] GuHCl dissolved in various solvents can alter the resultant volume, we found that to make up 500ml, dissolving 95.5g of GuHCl in 92ml of 1xTE gave a volume of 167ml (≈500/3).

by 80°C for 5’ to denature enzymes. The design of the adaptors was such that when ligated to the cut ends they did not recreate the restriction site.

Ligated fragments with mismatched ends were amplified by PCR using standard Illumina primers using Thermo-Fisher Phusion HotStart polymerase, using standard conditions and 20 cycles. The design of the adaptor sequences is such that only fragments containing a rare-cutter adaptor can be amplified by PCR, meaning amplification is limited to RC-RC and CC-RC fragments (Fig. 3.1). Due to the fact that RC-RC ended fragments and ligations of two rare-adaptors are able to amplify, it was necessary to titrate the amount of rare-cutter adaptor in the preceding step to minimise the presence of these fragments.

Barcoded adaptors consisted of 8 unique, doubly redundant barcodes each for the common and rare cutter adaptors, generated using the R package DNABarcodes (Buschmann & Hancock, 2017). Barcodes were 9bp long and featured a variable spacer sequence of 0-3 bp.

Figure 3.1 is a schematic of the priming sequence that ensures only ligated fragments with a rare-cutter adaptor amplify during PCR. The priming sequence for the common-cutter adaptor to anneal to the i5 Illumina indexing primer is not generated until the fragment has undergone one round of PCR from the i7 end.

Amplified samples were visualised on an agarose gel to check for amplification, and then cleaned using a double size-selection with AMPure XL beads. Cleaned PCR product was then quantified using the Fragment Analyzer to check the concentration of fragments within the target size range (300-700bp), pooled and size selected by PippinPrep, and sequenced on the Illumina HiSeq using S4 chemistry.

Figure 3.1–- Priming sequence for ligated GBS fragments. Bases in purple are the capture ends of the Illumina adaptors; red bases are Illumina index regions; yellow are the priming ends of Illumina indexing primers; green are the priming ends of the adaptors; blue Ns are the barcodes; black are the restricted fragments

3.3.5 Sequence processing

Barcoded DNA sequences obtained from the Centre for Genomic Research (CGR) were demultiplexed using the program cutadapt 4.0 (Martin, 2011). Samples were provided already demultiplexed at the index level, and were further demultiplexed in two stages, first by the rare-cutter adaptor and then by the common cutter adaptor, with the arguments *-e 0.15* and*–-no-indels*.

Sequences were then aligned to a reference genome constructed by Dr Raj Whitlock, based on a diploid individual of *F. ovina* from Öland in Sweden. Alignment was done using the program hisat2 2.1.0 (Kim *et al.*, 2019), and piped to SAMtools 1.15.1 (Danecek *et al.*, 2021), then filtered for quality and merged with the following code.

```
Hisat2--score-min L,0,-0.8--no-softclip--no-spliced-alignment
-5 3 -3 3 -I 60 -X 700 [input files, read groups and
reference genome] | samtools view -b | samtools sort -m 5G -@
90 -o [output BAM file]

samtools view -q 30 -b [input BAM] > [q30 BAM]

samtools index [q30 BAM]

samtools merge -c -@ 40 -o [merged BAM] [all q30 BAMs]
```

The merged BAM files were then analysed for variants using the programs sambamba 0.8.2 (Tarasov *et al.*, 2015), freebayes 1.3.6 (Garrison & Marth, 2012) and GNU parallel (Tange, 2011). To make efficient use of processing power and enable parallelisation, a balanced target regions file is created, as suggested by Garrison (2018), using *sambamba depth base–-combined* to generate a file of the base-by-base read depth.

As the data had high variability in coverage, some basic pre-filtering was applied to the coverage file using *awk* to remove any bases with coverage < (min coverage per pool * number of pools). In addition, the BEDtools suite of programs (Quinlan & Hall, 2010), specifically *bedtools intersect* was used to pre-filter the BAM file to exclude repetitive

regions as these may have high coverage while being uninformative. With these preparations, the following code was run to call variants.

```
Cat [target regions] | parallel -j 100 freebayes -0 -f
[reference genome] -b [merged BAM] -K--min-coverage 48 -G 5 -
F 0.01 -C 2--limit-coverage 960 -g 100000--region {}‘’’’
./tmp/{}.vcf

cat [regions file] | while read a; do echo ${a}.vcf; done >
./tmp/concatfiles

cd ./tmp; cat $(cat concatfiles) | vcffirstheader |
vcfstreamsort -w 1000 | vcfuniq > [merged vcf]
```

The resultant VCF file was filtered using BCFtools 1.15.1 (Danecek *et al.*, 2021) using *bcftools filter* with the parameters quality >1, total read depth >1000 and reads supporting alternate alleles to the left and right >1. Due to the requirements of downstream statistical software, *bcftools view -m2 -M2* was used to leave only biallelic loci. Low quality pools and loci, i.e. those with low depth or large amounts of missing data, were identified using *vcftools* and removed from the analysis.

The final set of pools that passed this step consisted of 18 pools, with 15,441 SNP loci with a minimum depth of 100 and no missing data.

3.3.6 Outlier identification

VCF files were converted to pooldata format using the R (R Core Team, 2020) package poolfstat (Gautier *et al.*, 2022), using the function *vcf2pooldata*. Analysis was then performed using Baypass 2.3 using the covariate model, coding slope aspect as an environmental and contrast factor. The covariate model in Baypass takes a pooldata file as an input, alongside an *efile* and a *contrast* file. The *efile* denotes the environmental factors, in this instance coding slope aspect as a binary factor of -1 and 1 for each pool represented in the pooldata file, the *contrast file* is similarly coded with -1 and 1 for slope aspect.

The covariate model outputs a matrix called Omega, a scaled covariance matrix of population allele frequencies across all SNPs. This Omega matrix is decomposed via the R function *svd()* which computes the singular-value decomposition of the matrix, and used to create a principal component analysis for inferring the levels of differentiation between populations. Other manipulations of the Omega matrix, such as *cov2cor()* are used to generate a correlation matrix, again based on the sum total of allelic frequencies across all populations. From this I was able to generate phylogenetic trees using *hclust()* to further infer demographic history.

In addition to the Omega matrix, using the covariate model with the *efile* and *contrast file* outputs two additional statistics used in this study, with values for each SNP in the input. The first is the XtX statistic, which highlights loci with allele frequencies different from the general population structure (Günther & Coop, 2013), with associated p-values denoting significance. The second is the Bayes factor comparing the likelihood that the allelic frequencies for individual SNPs are associated with the provided environmental factor (here: slope aspect) vs the null model.

Pairwise and total F_{ST} values for SNPs were generated by the poolfstat functions *computePairwiseFST()* and *computeFST()* with the method "anova". SNP significance was assessed by Bayes factor score ≥ 20, a p-value for XtX stat of ≤ 10^{-5} for the contrast analysis and an F_{ST} score ≥ 0.15.

Outlier SNPs were extracted to a subset VCF file and annotated with a GFF file provided by Dr Raj Whitlock, with the tool *vcfanno* to quickly identify SNP loci that fell within identified genes (Pedersen *et al.*, 2016). Loci identified as significant that failed to align with any annotations were further identified by using NBCI's online tool nucleotide BLAST tool, using the 200bp sequence either side of the SNP.

3.4 Results

The results suggest a great deal of genetic differentiation exists between populations. Only a relatively small proportion of which can be ascribed to slope-aspect microclimate, however there are loci that show strong evidence for selection that align with slope-aspect microclimate.

3.4.1 Outlier identification and population structure

Figure 3.3. shows the calculated F_{ST} values for all biallelic SNPs that passed filters. Of the 15,441 SNPs, 557 (3.6%) had F_{ST} values over the accepted cut-off value signalling selection of 0.15. Fourteen of those had a p-value ≤ 0.00001 when contrasted between slope aspects, highlighted in pink. Bayesian analysis using slope-aspect as a predictive factor identified eight SNPs with a bayes-factor above 20 and an F_{ST} value over 0.15, marked with blue triangles. The combination of these two statistics gives us a total of 17 SNPs associated with slope-aspect, with a core of 5 SNPs identified by both tests. Mean F_{ST} values were 0.025 for putatively neutral SNPs and 0.247 for outliers (ANOVA F = 399.2; P-value = < $2.2x10^{-16}$).

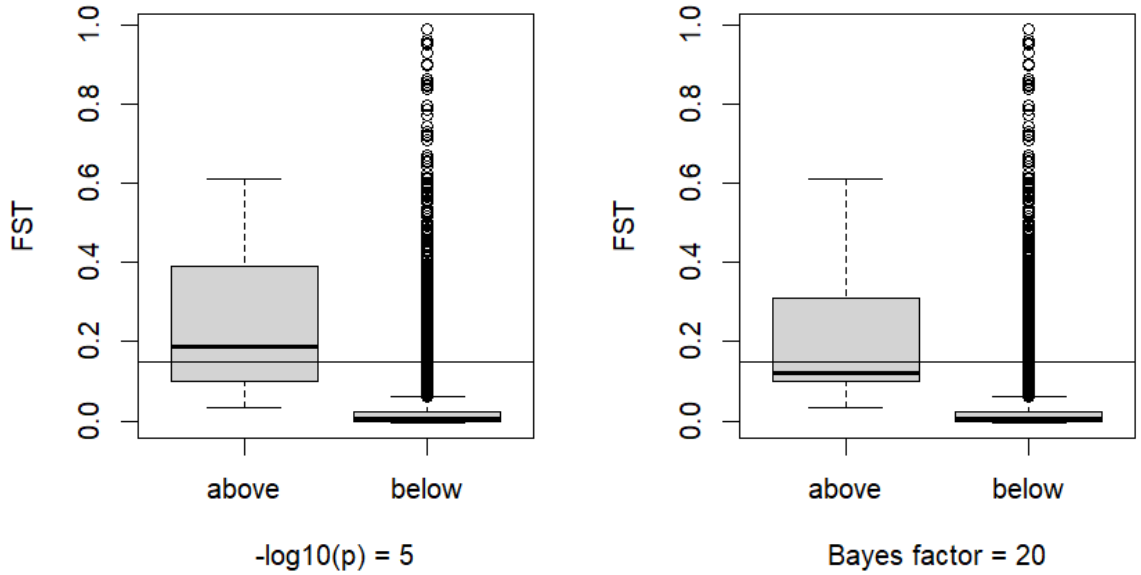


Figure 3.2 – Boxplots of F_{ST} values for SNPs with significant (above) or insignificant (below) values from BayPass analysis of contrasts (left) and environment (right). The horizontal line marks F_{ST}=0.15. Significance was defined as a -log10(p) value of ≥ 5 or a Bayes factor score of ≥ 20. The distribution of SNPs recovered as significant through Bayesian analysis was skewed heavily towards higher F_{ST} values than the majority of SNPs, supporting the idea that they are under selection vs. putatively neutral loci.

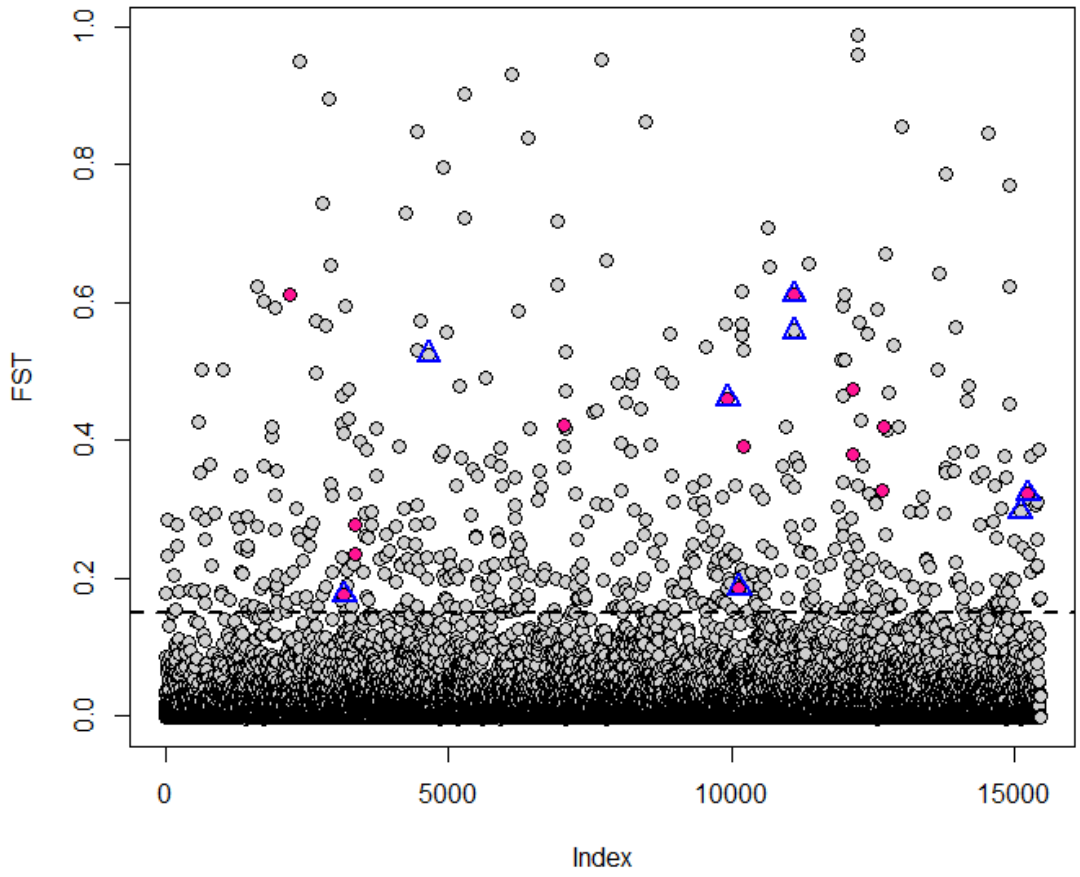


Figure 3.3 – SNP F_{ST} values from poolfstat. The index is a sequential numbering system representing each SNP as a point on a list. The dashed horizontal line marks F_{ST} 0.15. Highlighted are 14 SNPs with a -log10 p value ≥ 5 (pink) and 8 SNPs with a Bayes Factor score ≥ 20 (blue triangle), that fall above F_{ST} 0.15. For readability and relevance, SNPs reaching either significance criterion but falling below F_{ST} 0.15 were not highlighted.

Figure 3.2 shows distribution of F_{ST} values for SNPs marked as significantly differentiated according to the two tests. A number have an overall F_{ST} value below 0.15, however these were excluded from further analysis. A correlation matrix (Fig. 3.4) identified a pattern of distinctly different sites, consisting of S001, S002, S012 and N015, which retained their outlier statue at all read depths checked. The remaining sites formed a cluster, as can be seen in the following PCA graphs.

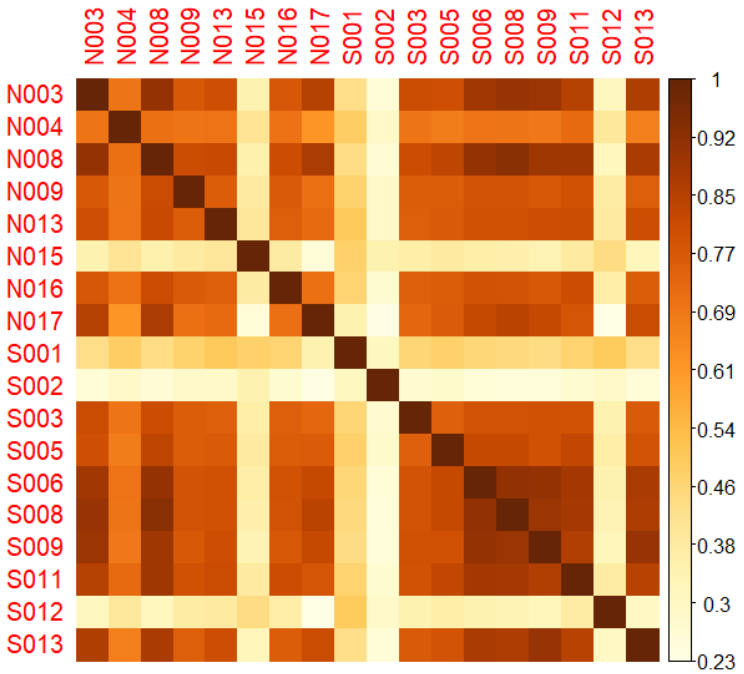


Figure 3.4 – Correlation plot of all sites, derived from the covariance omega matrix from BayPass illustrating overall high levels of covariance between sites from both north and south facing aspects, with the exceptions of sites S001, S002, S012 and N015

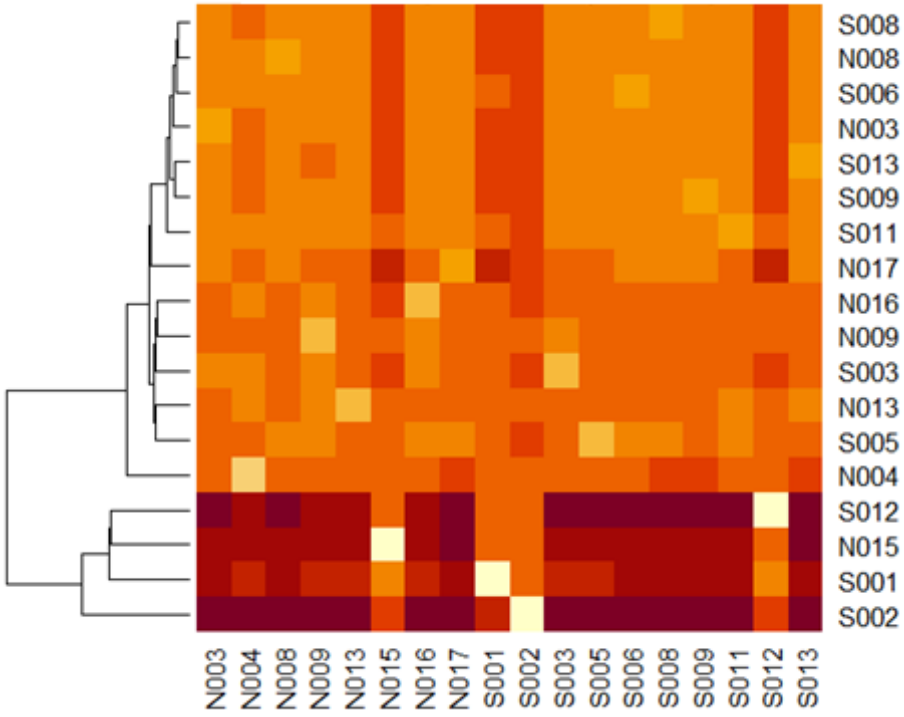
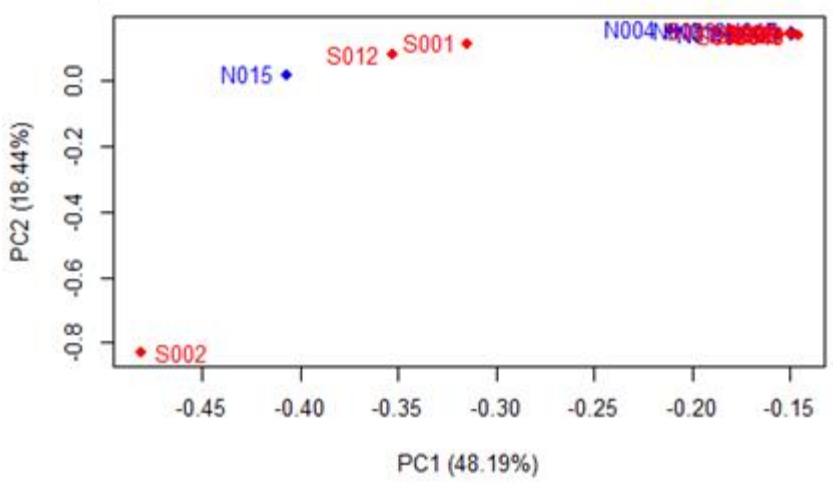
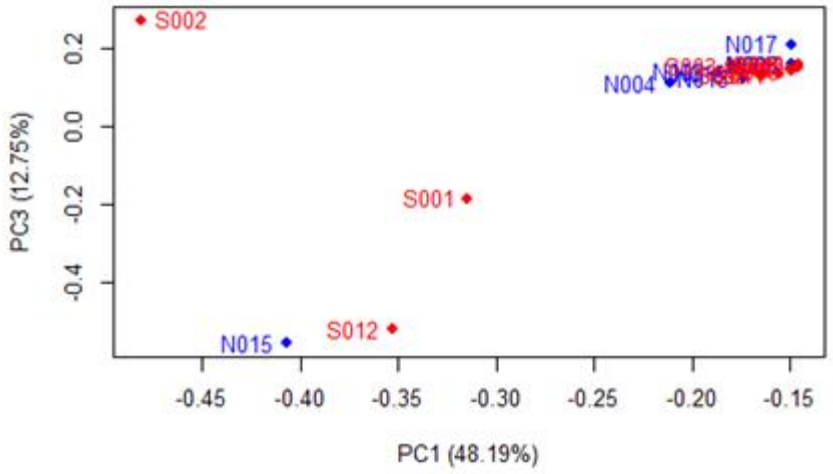


Figure 3.5 – Heatmap diagram of distance matrix derived from BayPass omega matrix across all SNPs, lighter colours are more similar, on the right is a tree of similarity using hclust(), *branch length indicating relative distance. Vertical axis is reordered in line with* hclust() *grouping, horizontal remains alphabetical.*





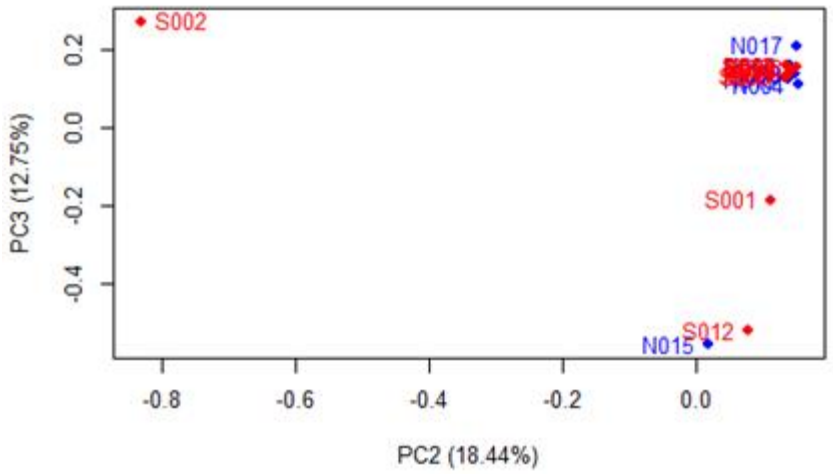


Figure 3.6 displays the results of the analysis performed with BayPass as PCA plots, show that much of the difference appears driven by a few populations, as visible in the correlation plot (ultimately derived from the same matrix). Other sites tend to be clustered relative to them, falling into several smaller groupings, as per the phylogenetic tree (Fig. 3.5.) in the heatmap diagram (Fig. 3.5.).

Figure 3.6 – PCA plots of the omega covariance matrix from BayPass, plotting PC's 1, 2 and 3 against each other. South facing slopes are marked in red, and north facing in blue. Data dispersal did not allow for neat labelling but was included to highlight outliers.

3.4.2 Location of outlier SNPs in genes and putative function

Of the 17 loci, 6 were found within genes according to annotation provided by Dr Raj Whitlock (table 3.2). Only two of the highly significant SNPs were located in these genic regions – *FEOV00050147*, a protein of unknown function and *FEOV00050338*, an enzyme involved in pectin synthesis.

*Table 3.2 – Gene IDs, positions and F_{ST} values for 17 of the identified SNPs associated with slope aspect. * denotes support from Bayes factor score above 20, † denotes support from contrast analysis p value ≤10^{-5}.*

SNP ID	Gene	Contig	Position	F_{ST}	Notes
2194		323,628	1,049	0.611	†
3160		475,332	13,148	0.175	†*
3341		483,019	649	0.277	†
3344		483,019	662	0.234	†
4648	*FEOV00058947*	549,210	1,079	0.524	Similar to CDF2: Cycling DOF factor 2 (*Arabidopsis thaliana*) *
7045	*FEOV00039041*	603,983	302	0.423	Similar to OsI_14861: Senescence-specific cysteine protease SAG39 (*Oryza sativa subsp. indica*) †
9932	*FEOV00050147*	635,547	3,353	0.460	Protein of unknown function †*
10130		637,609	15,916	0.186	†*
10196	*FEOV00017574*	637,788	85,999	0.391	Similar to RGA5: Disease resistance protein RGA5 (*Oryza sativa subsp. japonica*) †
11102		642,162	241,537	0.612	†*
11103		642,162	241,538	0.558	†
12144		647,066	223,055	0.380	†
12149		647,112	28,751	0.474	†
12642	*FEOV00022221*	648,835	50,421	0.327	Similar to Rf1: Protein Rf1, mitochondrial (*Oryza sativa subsp. indica*) †
12691		648,958	84,646	0.420	†
15093		658,150	96,605	0.296	*
15225	*FEOV00050338*	672,051	479	0.322	Similar to RHM2: Trifunctional UDP-glucose 4,6-dehydratase/UDP-4-keto-6-deoxy-D-glucose 3,5-epimerase/UDP-4-keto-L-rhamnose-reductase RHM2 (*Arabidopsis thaliana*) †*

The gene *FEOV00050338* is likely a trifunctional enzyme involved in the synthesis of rhamnose and the pectin elements of cell walls. Gene *FEOV00017574*, likely coding for a disease resistance gene associated with fungal pathogens such as *Magnaporthe grisea*, had a high p-value in the contrast analysis ($10^{-6.97}$) but only a moderate Bayes factor score (13.3).

FEOV00039041 is similar to the mitochondrial gene *Rf1* in rice, which is responsible for control of male cytoplasmic sterility, as well as editing of mRNA associated with ATP synthase. *FEOV00058947* shows similarity to *CDF2* from *A. thaliana*, part of a gene family of transcriptional regulators responsible for mediating flowering response to day length (Sun *et al.*, 2015). *FEOV00039041* is similar to *OsI_14861*, a proteolytic enzyme of the lysozyme associated with senescence and the hypersensitive response to infection.

Using NBCI's BLASTN on sequences flanking unannotated SNPs identified possible associations between some of them and known or putative sequences from other plants. SNP 2194's flanking sequences returned a match for a predicted *Brachypodium distachyon CSC1-like* protein (*At3g21620*, NCBI ref: XM_024460063.1). SNP 3160 matched to a number of BTB/POZ and MATH domain-containing protein 2-like sequences from grain crops (e.g. NCBI ref: XM_048681947.1).

The pair of SNPs 3341 and 3344 were spaced 13bp apart, however did not appear located near any known or predicted genes. 10130 has some similarity to a predicted translation initiation factor 2 from *B. distachyon* (NCBI ref: XM_024462084.1). SNPs 11102 and 11103, adjacent nucleotides, are within 200bp of *Triticum aestivum* uncharacterized LOC123082883, derived from mRNA sequencing. SNP 12149's flanking sequenced placed it in a predicted *T. aestivum* F-BOX protein (*At5g03970*-like, NCBI ref: XM_044589869.1). SNP 12144 was located in a sequence with similarity to a ncRNA identified in *Hordeum vulgaris*, the function of which is unknown. The sequence containing SNP 15093 aligned on blast with *H. vulgaris* phosphate transporter 1 (*PHT1*).

3.4.3 Pairwise F_{ST} between sites

Pairwise F_{ST} calculations (table 3.3) for all sites agreed with the distance matrix PCA generated by BayPass (Fig. 3.5). Sites S001 and N015 were significantly differentiated from all other sites and each other, and S012 and N004 both showed strong differentiation from most other sites. Mean F_{ST} for comparisons between slope aspects was 0.116, while the

mean of values within slope aspect 0.115; there was no significant difference in F_{ST} values for within vs between sites (ANOVA F = 0.0285; P= 0.9719).

Table 3.3 – Pairwise F_{ST} values for sites across all loci from poolfstat, scores ≥ 0.15 are highlighted in red.

N004	0.152																
N008	0.021	0.139															
N009	0.076	0.182	0.060														
N013	0.046	0.156	0.035	0.081													
N015	0.204	0.311	0.187	0.238	0.206												
N016	0.066	0.163	0.050	0.088	0.071	0.223											
N017	0.028	0.148	0.023	0.074	0.045	0.178	0.063										
S001	0.183	0.287	0.173	0.210	0.174	0.350	0.197	0.164									
S002	0.143	0.253	0.137	0.187	0.147	0.230	0.166	0.120	0.252								
S003	0.047	0.168	0.050	0.093	0.068	0.222	0.083	0.057	0.199	0.160							
S005	0.048	0.160	0.033	0.079	0.057	0.198	0.065	0.040	0.194	0.149	0.066						
S006	0.026	0.149	0.020	0.074	0.045	0.202	0.060	0.027	0.184	0.143	0.057	0.041					
S008	0.021	0.153	0.014	0.070	0.044	0.199	0.060	0.023	0.183	0.138	0.054	0.040	0.017				
S009	0.024	0.157	0.026	0.078	0.045	0.202	0.067	0.028	0.179	0.140	0.056	0.047	0.020	0.022			
S011	0.038	0.142	0.024	0.071	0.045	0.196	0.055	0.039	0.177	0.143	0.061	0.038	0.027	0.029	0.037		
S012	0.168	0.282	0.157	0.208	0.178	0.309	0.190	0.149	0.318	0.212	0.189	0.172	0.169	0.162	0.165	0.163	
S013	0.033	0.153	0.029	0.078	0.042	0.196	0.065	0.032	0.171	0.129	0.059	0.048	0.027	0.029	0.020	0.035	0.164
	N003	**N004**	**N008**	**N009**	**N013**	**N015**	**N016**	**N017**	**S001**	**S002**	**S003**	**S005**	**S006**	**S008**	**S009**	**S011**	**S012**

3.5 Discussion

In this chapter I analysed the genetic differentiation between sites in the Peak District, using GBS to identify SNPs under selection. The aim of this was to answer the question of whether microclimate caused by slope aspect drives changes in genotype between populations of *F. ovina* from north and south facing slope aspects. The main findings of this study were that though overall differentiation between fourteen of the eighteen sites in this study was low, with no evident population structure related to slope aspect, there was strong evidence that specific loci were under selection for slope aspect. While pairwise F_{ST} comparison indicated little overall separation or reproductive isolation between most populations, a small collection of loci showed strong signs of selection for slope aspect. Loci under selection were associated with genes related to transcriptional regulation, timing of flowering, water stress response and disease resistance. In addition, certain populations were resolved as significantly distinct from most or all others, for reasons unrelated to slope aspect.

Using a pooled genotyping-by-sequencing approach (Bélanger *et al.*, 2016) and Bayesian analysis of the resultant sequences, I was able to identify 15,441 biallelic SNPs with

sufficient depth of coverage to estimate their allele frequencies across eighteen populations of *F. ovina* from the Peak District National Park in England. Seventeen of the SNPs identified in this study showed statistically significant differentiation between populations from north and south facing slope-aspect microclimates.

Four of the five loci with the highest levels of statistical support appeared to be located in genes for response to osmotic stress, a translation initiation factor, pectin synthesis and one of unknown function. The three with known functions all have putative links to abiotic stress responses supporting their candidacy as being under selection due to microclimate. SNP 3160 is located in a region homologous to other grass BTB/POZ-MATH protein 2-like sequences. The function of the BTB/POZ-MATH (BPM) family of proteins in *A. thaliana* have been linked to cellular response to water deprivation and osmotic stress, being upregulated when subjected to these stresses (Weber & Hellmann, 2009). SNP 10130 is likely located in a gene coding for a protein similar to transcription initiation factor 2 (IF2-like), a class of proteins found across all domains of life with a role in the assembly and maturation of ribosomes. Potentially relevant to discussion of microclimate is their role in cold-shock response in bacteria – IF2 is upregulated in response to cold shock in *Escherichia coli*, with IF2 mutants showing cold-sensitivity and accumulation of immature ribosomal particles (Brandi *et al.*, 2019; Laursen *et al.*, 2003). Gene FEOV00050338 is putatively similar to an *A. thaliana* gene with several enzymatic actions related to pectin synthesis, important in cell wall development and mucilage production. Mucilage production around the root cap and rhizosheath is associated with, and altered by, drought response in grasses, again supporting the gene's involvement in adaptation to slope aspect microclimate (Chaffey, 1996; Galloway *et al.*, 2020; Usadel *et al.*, 2004). Supporting this are reports of rhizosheath composition in the desert grass *Stipagrostis pungens* containing rhamnose (one of the products of *RHM2*) (Marasco *et al.*, 2022) and in switchgrass *Panicum virgatum* (Liu *et al.*, 2019). In the Liu *et al.* study, a greater increase in rhizosheath weight was seen the drought-adapted Alamo genotype compared with the more wet-adapted Kanlow genotype.

Eight of the SNPs with lower, but still significant, statistical support for association with slope-aspect microclimate could be a linked to known genes in *F. ovina* or other plants. *FEOV00058947* is similar to a protein in *A. thaliana*, *CDF2*, a transcription regulator involved in the sensing of day length and possibly floral initiation (Fornara *et al.*, 2009; Sun *et al.*,

2015). A potential role for this gene in slope-aspect microclimate adaptation is in an alteration of flowering time. Plant populations on south facing slopes in the northern hemisphere may flower earlier than those on north facing, as in *Fritillaria unibracteata* (Chen *et al.*, 2016) and 27 species in Sonoma County, California (Olliff-Yang & Ackerly, 2020). Climatic adaptation to drought in *F. ovina* has been demonstrated to alter flowering time and early-acting life history traits (Trinder *et al.*, 2020). The increase in summer drought stress on south-facing slopes (Rorison *et al.*, 1986), may select for earlier flowering and seed maturation in populations on these slopes.

One locus may have a role in disease resistance, *FEOV00017574* is similar to the protein RGA5, involved in defence against a common fungal pathogen of graminoids (Ortiz *et al.*, 2017). As infection rates by *M. grisea*, a well-studied crop pathogen, vary with both temperature and humidity, differing pathogen pressures driving differentiation between slope aspects is plausible (Alves & Fernandes, 2006; Roumen *et al.*, 1997). *FEOV00039041* is similar to sequences for SAG39, a multifunctional proteolytic enzyme characterised as a senescence associated gene. SAG enzymes are important in the remobilising of nutrients during leaf senescence but also have diverse other functions, including in drought sensing and the hypersensitive response to infection (Sarwat *et al.*, 2013; Wehner *et al.*, 2016). As such there are many potential roles that could explain selection for different alleles between north and south facing slopes.

SNP 12642 was located in gene *FEOV00022221*, which encodes a mitochondrial Rf1 like protein. The Rf family are best known for their role in counteracting male cytoplasmic sterility, but are part of a larger group of proteins that control the expression and transcription of many proteins through cleavage of mRNAs. There is no indication of the specific mechanism by which this gene may be selected for by climate, but other, related genes have been implicated in promoting resistance to abiotic stresses (Li *et al.*, 2021). Similarly, SNP 12144 appears in a sequence similar to a barley ncRNA of unknown function, however ncRNAs in plants are commonly known as regulators of gene expression.

The final two loci are SNP 2194, located in a sequence that appears to be a *CSC1-like* protein and 15093, in a sequence similar to a *H. vulgaris PHT1* gene. The *CSC1* gene in *A. thaliana* is an osmosensitive calcium permeable cation channels thought to influences drought response. Other members of this family are implicated in long-distance signalling for

stomatal closure in conditions of hyperosmolarity, indicating a potential role for this gene in microclimate adaptation between slope aspects (Hou *et al.*, 2014).

PHT1 is a phosphate transporter involved in plant uptake of inorganic phosphate from the soil (Nussaume *et al.*, 2011). Phosphate is known to have poor availability to plants in both acidic and basic soils, with a peak availability between pH 5 and 6. In the calcareous soil of CG2 grassland, phosphate availability is known be a limiting factor in the growth of *F. ovina* and drought survival in the grass *Arrhenatherum elatius* (Grime & Curtis, 1976). Phosphate availability and absorption in plants, including graminoids, varies with soil temperatures (Chapin, 1974; Chapin & Bloom, 1976) and soil temperature varies significantly between north and south facing slope aspects in the Peak District (Rorison *et al.*, 1986). Drought conditions are also implicated in reducing phosphorus availability in soils (Sardans & Peñuelas, 2004; H. Zhang *et al.*, 2020). As it's an established limiter of growth in *F. ovina* at Peak District CG2 sites, and likely even more limiting in more drought prone sites, evolutionary change in phosphate transporter 1 and similar genes may be expected between microclimate populations.

This study was not without its limitations, and has potential for further development. Issues with the formulation of the common cutter adaptors for several of the barcodes led to poor amplification for a number of samples, reducing available data for use. Information on soil depths as a source of confounding variation for all sites may have improved resolution of data, due to its known effects on microclimate adaptation and drought stress known from the Peak District and studies at the BCCIL (Grime & Curtis, 1976; Ravenscroft *et al.*, 2014). Better quality of sequencing might have yielded more SNPs and loci under selection for slope aspect. Despite these shortcomings, the work presented here still finds strong evidence that slope aspect microclimate drives genetic differentiation for genetic traits and provides an important source of climatically adaptive variation. The data also suggests the importance of selecting good numbers of replicate sites in identifying evolution due to climatic variation. The sites S001, S002, S012 and N015 were strongly differentiated to all sites, irrespective of slope aspect; including one or two of those in a study consisting of only four sites would have the potential to skew results.

Lacking detailed histories of all slopes in this study I can only speculate on explanations for the high levels of divergence seen for sites S001, S002, S012 and N015. The vegetation

found at site S002 was dominated by *Helianthemum nummularium*, and a deeper bryophyte later than I normally observed on south facing slopes; similarities between the vegetation and morphology of *F. ovina* plants from S002 and north facing slopes is discussed further in chapter 2. The shorter morphology and overall sward characteristics for site S001 are also covered in chapter 2. S012 and N015 were not noticeably distinctive nor geographically distant from other sites that don't register as such extreme outliers that might explain this difference. Alternatively, it is possible that these samples were somehow mishandled, e.g. an error in pooling individuals, leading to a strongly biased allele ratio in the final pool. However, in light of the noticeable morphological differences identified for two of these site in chapter 2, it seems likely that at least S001 and S002 represent populations that are genuinely genetically divergent from others, rather than an artefact of sample handling.

The low F_{ST} values between the majority of sites may be interpreted as indicative of continued gene-flow between populations involved in the study, however from a geographical perspective this seems unlikely as a primary explanation. For example, sites S003 and S005 have a pairwise F_{ST} of 0.066, but are separated by over 14km and are in different dale systems. As such this low F_{ST} is probably not indicative of regular, mass gene flow between these two populations. Rather, it is more likely a product of *F. ovina*'s mating system favouring the retention of genetic variation, even in smaller, fragmentary populations (Berge *et al.*, 1998). In contrast, S005 forms a perfect north-south pair with N004 – there's less than 100m between them and they have a pairwise F_{ST} value of 0.160. Gene-flow events between these two slopes almost certainly occur with some regularity, however the two present a very extreme hot and dry vs. cool and wet contrast when viewed in person; differences between plants collected from the two sites were also pronounced for growth (2.4.1) and leaf tissue traits (2.4.2), (but not flowering traits (2.4.3), interestingly), in the phenotypic study of chapter 2. With the large effective population size characteristic of these sites and the reproductive biology of *F. ovina*, F_{ST} may be less indicative of gene-flow at these geographic scales (see Bengtsson *et al.* (2004)), instead suggesting certain populations may have been subject to particularly strong selective pressures or selective sweeps at some point in their history. E.g. whatever has driven the dwarfing of S001, or, hypothetically, selection for reproductive isolation between S005 and N004 due to their close proximity and highly contrasting environmental characteristics.

In conclusion, the data presented here, in agreement with the findings of the previous chapter, supports the potential role of slope-aspect microclimate and individual sites as sources of important adaptive genetic variation in *F. ovina*. Genotypic data supports the hypothesis that fine-scale, microclimatic variation drives ecologically adaptive genetic change at an equally fine spatial scale. Furthermore, it provides evidence that abiotic conditions or microclimate-associated shifts in the biotic environment are driving this observed differentiation between slope aspects. And finally, the presence of microclimatic variation such as this may foster the evolution and maintenance of climatically adaptive genetic variation in the landscape, with the potential to buffer these populations against the effects of anthropogenic climate change.

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4 The genetic response of *Festuca ovina* to 25 years of three climatic manipulations at the Buxton Climate Change Impacts Laboratory

4.1 Abstract

Environmental manipulation studies have long been a vital part of ecological research, providing insight into *in situ* effects of climatic and edaphic factors on plant communities and evolutionary processes. Climate manipulation at the Buxton Climate Change Impacts Laboratory (BCCIL) has been running for 29 years, providing a wealth of information relating to the impacts of climate change on plant community structure, species abundance, invasion, evolutionary responses and plant-soil interactions (Bates *et al.*, 2005; Ravenscroft *et al.*, 2014, 2015; Sayer *et al.*, 2017). Experimental systems such as this are important (i) because the diverging properties of plant populations can be directly attributed to simulated climate change and (ii) because they can give us insight into the fundamental processes driving evolutionary changes, including the identity of loci under selection. Although genetic responses to simulated climate changes have been documented within natural ecosystems in a handful of studies, the genomic basis of these effects have been investigated only very rarely. Here I applied genotyping-by-sequencing to populations of the grass *Festuca ovina* from different simulated climate change treatments at BCCIL to explore the genomic basis of previously documented phenotypic and genetic differentiation driven by the treatments. I documented a pattern of ecologically adaptive genomic differentiation in a small subset of loci, linked with both experimental summer drought and winter warming treatments. Contrary to the findings of previous molecular studies my results show that the warming treatment drove a greater degree of genetic divergence from control populations than the drought treatment, although both treatments exhibited significant signals of selection relative to control conditions and to each other. The putative functions of genes harbouring significantly differentiated GBS SNP Loci indicate that responses to climatic selection involves traits related to pollen-formation, heat stress tolerance and the sensing or response to day length. Comparison of these results to those observed in relation to variation in slope-aspect microclimate, found overlap in the types of gene function, but not in gene identity. In other words, experimental climate treatments and natural

microclimatic variation appear to select for genetic change in the same types of gene function, but through different gene loci. The results provide evidence that responses to selective pressures from climate manipulation at the BCCIL are indeed functionally comparable to those occurring at naturally occurring microclimate sites in the wider landscape.

4.2 Introduction

Climate change over the coming century is predicted to subject plants to strong selection pressures (Coleman *et al.*, 2020; Cunze *et al.*, 2013; IPCC, 2014) and risk of extinction (Jump & Peñuelas, 2005; Myers *et al.*, 2001). Ecologists and conservationists aim to make predictions as to these effects, often with the hope of mitigating damage or devising alternate conservation strategies (e.g. *ex situ* conservation interventions). Vital to this process is evidence for the how plant communities respond to alterations in climate, which is largely gathered from observational studies using space-for-time approaches, and from direct experimental manipulation of plant communities.

Climate manipulation studies are widely recognised as a valuable tool in understanding evolutionary processes affecting plant communities. The Rocky Mountain Biological Laboratory is the world's oldest continually running climate manipulation experiment, established in 1990 and situated in a subalpine meadow in Gunnison County, Colorado, USA. Heating trials over a period of 4 years (1991-1994) demonstrated changes in aboveground biomass, with an increase in shrubby biomass and decrease in forb biomass. Their results suggested even small changes to soil temperature (max observed: +1.6°C), date of snowmelt (max observed: -15 days) and/or soil moisture % (max observed: -2.7%) have the potential to shift the community composition significantly (Harte & Shaw, 1995). The experimental heating was continued, and over the first 10 years demonstrated a significant loss (an average 22% reduction) of soil-organic-carbon in the heated plots relative to the control plots (Saleska *et al.*, 2002). Between 2002-2005 the experimental area experienced a prolonged drought, with a loss of soil-organic carbon from the control plots comparable with that seen in the first 5 years of the heated treatment, forming an invaluable comparison between the results of experimental work and naturally occurring climatic conditions as a result of climate change (Harte *et al.*, 2006). Another, shorter-term (3 years)

climate manipulation study combining translocation of ecotypes of the grass *Festuca lenensis* on the Mongolian steppe, modifying precipitation and temperature, found strong plant-plant interactions influencing response to climate, but also strong effects of ecotype, evidencing the importance of local variation in driving community response to climate (Liancourt *et al.*, 2013).

The longest running climate manipulation study in Britain is the Buxton Climate Change Impacts Laboratory, established in 1993 by J. Phil Grime of the University of Sheffield. Consisting of 5x replicates of soil heating, precipitation alteration, control and factorial plots, on a species rich grassland, the site has provided a wealth of studies on the effects of climate change on community composition, species abundance, evolution and genetics. Bryophyte cover was found to be reduced after seven years of drought treatment, with specific reduction in two species, and increase in one; winter warming (+3°C soil temperature between November and April), saw increased cover of one species, but reduction in two others and overall loss of species diversity (Bates *et al.*, 2005). Early analysis, after five years, comparing the site with the early successional post-arable calcareous grassland at Wytham (Oxfordshire), indicated that grasslands vary in their resistance to climate change. The community at Wytham was far less stable in response to altered climatic conditions compared to the stable old sheep pasture dominated by stress-tolerators at Buxton (Grime *et al.*, 2000). This difference in resistance to climate change though raises questions about the extrapolation of data drawn from specific manipulation experiments to wider systems. Changes are evident at both the species and higher taxonomic level, while demonstrating a remarkable overall stability as few species are actually lost from the site.

Later work at BCCIL has focused on the evolutionary effects climate manipulation has had on species, *F. ovina* and *Plantago lanceolata*, two abundant species across all treatments at the site. An array of F1 crossed progeny of *F. ovina* collected from the drought and control plots, after 17 years of treatment, demonstrated heritable changes in germination time between the treatments. Plants from the drought treatment flowered on average 1-3 days earlier than plants from the control treatment, leading to a level of assortative mating driving differentiation between the populations (Trinder *et al.*, 2020). *P. lanceolata* populations from control and drought treated plots were found to differ in traits related to

competition vs reproduction after 15 years of climate manipulation, when grown in a common garden. Droughted plants favoured allotting resources to sexual reproduction (drought avoidance), while plants from the control plots invested more heavily in competitive traits (Ravenscroft *et al.*, 2014). Notably they also found edaphic conditions, (heterogeneity in soil depth is high at the site) to strongly influence the relative responses of the plants to the treatment. A related study focusing on *F. ovina* and *P. lanceolata* was done after 16 years of treatment, using AFLP to assess genetic divergence and diversity between the plots. Using 360 individuals from each species (12 individuals each from 5 replicates of 6 treatments) all treatments were found to differ significantly from control plots (Ravenscroft *et al.*, 2015). Taken together these studies suggest habitats heterogeneous for edaphic and climatic conditions, such as that at the BCCIL, may be expected to contain appreciable adaptive diversity enabling evolutionary responses to rapid climate change.

Assessments of the effects of climate change on plants using space-for-time and climate manipulation experiments have been demonstrated to broadly agree on trajectory, while differing in magnitude of effect size (Elmendorf *et al.*, 2015). The metanalysis by Elmendorf *et al.* found that experimental and observational studies generally agreed on the magnitude of change, space-for-time tended to estimate greater changes in the relative abundance of species with a warmer thermal niche. Though areas where climate manipulation experiments are done are often also used in space-for-time analyses, few studies assessed the repeatability of genetic responses found between the two types of study. Further to that, most study sites demonstrate dramatic changes to the community structure in response to climate manipulation (Grime *et al.*, 2000; Harte & Shaw, 1995), in contrast to the BCCIL community which has proven remarkably resistant to change (Grime *et al.*, 2000, 2008). As it stands there is little research investigating the applicability of results gained from climate manipulation to naturally occurring sources of climatic variation, and little specifically regarding genetic differentiation compares between experimentally manipulated and naturally occurring populations in these resistant community types.

Understanding population genetics and evolutionary processes is vital to conservation interventions in a changing climate (Aavik & Helm, 2018; Neale, 2012; Thomas *et al.*, 2014). Studies on natural populations can only tell us so much about the response to sudden climate change, a potential weakness in employing the space-for-time paradigm as a

predictor of response to the forecasted rapid changes (IPCC, 2014) in the coming century. Climate manipulation experiments however can be argued to be *too* abrupt , compared to the expected rate of changes (Elmendorf *et al.*, 2015). In order to understand how best to protect and manage plant populations in the future it is important that we know not only how natural populations may be adapted to climate and how they react to sudden changes, but how those effects differ and relate to each other.

In this chapter I aim to answer the question of whether the genetic changes driven by climate manipulation studies are equivalent to those seen in naturally occurring microclimate population. I did this using the *F. ovina* population that has undergone 23 years of continuous climate manipulation for precipitation and winter temperatures in comparison with control plots and natural populations from a well-replicated, spatial mosaic of north and south facing topographical slope aspects and their associated contrasting climatic condition.

The BCCIL provides an invaluable study system due to its location on a west facing slope in the Peak District, as the landscape of the surrounding area featuring a number of deep dales featuring north and south facing slopes. As the previous chapter of this thesis investigated the effects of selection for slope aspect microclimate, using sites located 20km or less to the north, east and south of the BCCIL, this provides an unrivalled opportunity to compare the changes observed in the manipulated population at the BCCIL with the slope aspect microclimate populations of the wider Peak District. Building on the work of the preceding chapter, I subjected samples collected from three of the manipulation treatments at the BCCIL, alongside the control plots, from all 5 replicate blocks, to the GBS library preparation and sequencing. By analysing the data alongside the results gathered from the wider Peak District, as well as in its own right, I was able to compare my findings with previous work on the *F. ovina* population at BCCIL and contextualise the findings with regard to natural populations.

The primary hypotheses assessed in this chapter are (i) that the populations of *F. ovina* for at the BCCIL will be differentiated with regard to treatment and (ii) that genotypes more prevalent in treatments will align with those found in slope aspect populations from the wide Peak District. Regarding hypothesis (i) several previous studies done on the BCCIL show good evidence for the genetic differentiation of the heated, watered and droughted

treatments from control, though interestingly not statistically from each other (Ravenscroft *et al.*, 2015; Trinder *et al.*, 2020). Hypothesis (ii) is based on the assumption that due to proximity, vegetation characteristics and the "slope-aspect-neutral" nature of the BCCIL site as a west-facing slope, recurrent patterns seen in the wider peaks (i.e. chapter 3) should be represented here due to similarity of soil temperature and moistures as drivers of evolutionary change.

Here I, in agreement that previous studies, demonstrate that *Festuca ovina* populations at the BCCIL have undergone significant genetic differentiation in response to twenty-five years of climate manipulation. The most dramatic difference was a result of winter-warming treatment, compared with drought and control treatments. Furthermore, I find both similarities and differences between the types of mechanisms under selection between the BCCIL treatments and slope-aspect populations in the wider Peak District.

4.3 Methods

4.3.1 Climate manipulation treatments

The treatments used in this study consisted of +3°C winter-warming ("heated" - H), extended summer drought ("droughted" - D) and no manipulation ("control" - C). The site is split into 5 blocks (A, B, C, D and E) on a west-facing hillside situated on the site of the Health and Safety Executive at Harpur Hill Industrial Estate, just outwith Buxton, Derbyshire (SK053704). Each block is divided into nine 3x3m plots, with replicates of each treatment randomly arranged in each plot (Fig. 4.1.).

The site consists of an old sheep pasture, and has been in constant operation as a climate laboratory since 1993, when the major climate manipulations were established.

- The heated treatments consist of heating cables embedded in the soil of the plots that heat it to 3°C above ambient temperatures during the winter months.
- The drought treatments consist of rain-activated shelters that roll over the plots during July and August to simulate an extension/amplification of the normal summer drought.
- While not used in this study, there are also factorial treatments combining heating and watering treatments and heating and drought treatments.

A fourth treatment, supplemental summer rainfall ("watered"), consists of an irrigation system that raises water input to 20% above the long-term monthly average between June and September. However, due to issues with library preparation, this was removed from the final analysis.

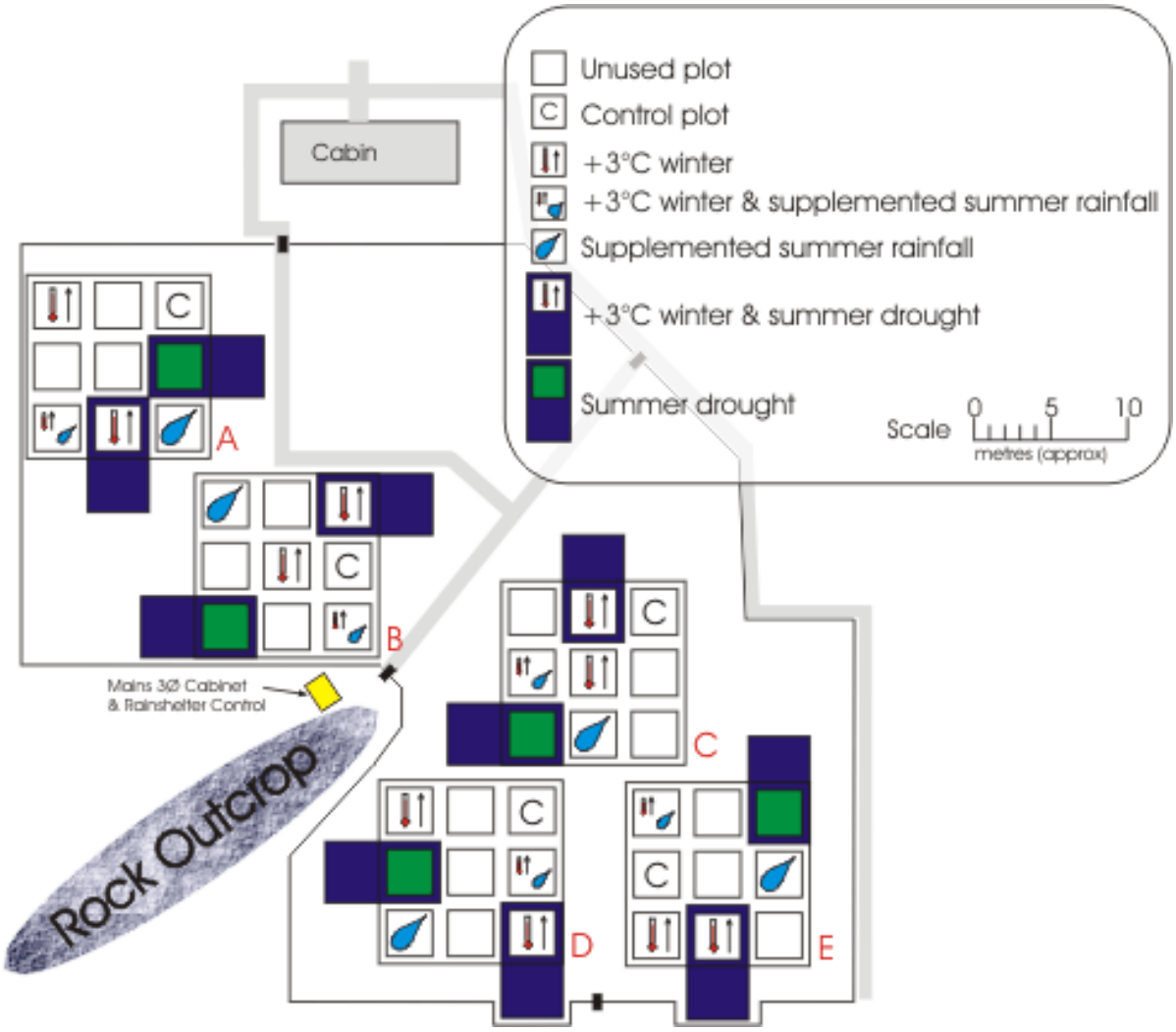

Figure 4.1- the plot layout of the BCCIL, taken from http://buxtonclimateimpacts.science/ For geographic location, see Fig. 1.1

4.3.2 Sample collection

Twenty-five samples were collected from each of the plots using a movable, 2x2m string quadrat, forming a grid of 5x5 points centred on the vertices (every 50cm). The size and positioning were designed to avoid edge effects, and to reduce the chance of collecting from the same individual twice. Samples were chosen as the *F. ovina* plant closest to the vertex but within 15cm, if no plant was located within a 15cm radius around the point, then

none was collected. Samples were collected in the form of 2-5 tillers, dependent on leaf length, with the aim of harvesting approximately 20-40mg of dry material. Tillers were stripped of dead leaves and leaf-sheaths before placing in screw-top, 2ml O-ring tubes filled ¾ with silica gel, an established method for preserving/preparing plant material for DNA extraction (Chase & Hills, 1991).

4.3.3 DNA extraction, library preparation and sequence processing

DNA extraction, pooling, sample handling and library preparation was handled as in chapter 3. DNA was extracted using a modification of the method described in Werth *et al.* (2016), pooled in equimolar concentrations and digested with the enzymes EcoRI-HF and HhaI-HF (NEB) and ligated to barcoded adaptors for amplification. Amplification was done using standard Illumina indexing primers for the HiSeq4000 platform, fragments were size selected for sizes between 300 and 700bp, and sequenced using S4 chemistry.

Sequence processing followed the same protocol outlined in chapter 3: Samples were demultiplexed using CutAdapt (Martin, 2011), and aligned to a draft genome compiled by Dr Raj Whitlock using the program hisat2 (Kim *et al.*, 2019). Alignments for all twenty samples were filtered to exclude repetitive regions using BedTools Intersect (Quinlan & Hall, 2010), merged into a single BAM file using Samtools (Danecek *et al.*, 2021) and variants called using FreeBayes (Garrison & Marth, 2012). Sequencing quality varied greatly, with a significant number of samples being excluded from the final analysis due to missing data and low read counts. Final samples with enough reads of sufficient quality, coverage and read depth for inclusion in final analysis consisted of two controls (blocks B and E), three drought treatments (blocks A, B and C) and two heated treatments (blocks A and E). Individual plots are labelled in the format Bx[block]_[treatment], meaning BxB_C is the control plot in block B, BxE_D is the drought plot in block E.

The samples for all watered treatments had to be excluded due to large amounts of missing data and low read counts. The final VCF consisted of 42,475 SNPs with a minimum and maximum read depth of 100 and 800, respectively, per sample and no missing data.

4.3.4 Outlier identification

Outlier loci were identified using the R (R Core Team, 2020) package *poolfstat* (Gautier *et al.*, 2022) to generate *pooldata* files from the VCFs, using the function *vcf2pooldata*.

Analysis was then performed using BayPass 2.3 (Gautier *et al.*, 2013). BayPass analysis was run in the covariate model (see section 3.3.6) with control, heated and drought treatments coded as environmental factors. Three contrasts were coded for comparisons: Contrast 1 - Control vs Drought; contrast 2 - Control vs Heated; contrast 3 - Drought vs Heated. Outliers were categorised as such with the combination of a p-value derived from contrasts ≤ 0.00001, $F_{ST} \geq 0.15$ and Bayes factor for the relevant environmental factor ≥ 20.

4.4 Results

No obvious pattern of population structure was found with regard to treatment where a single treatment clustered together neatly (Fig. 4.2). Heated plots had the greatest number of divergent SNPs relative to the other two treatments but also showed significant difference between the two heated plots. Principal component analysis based on singular value decomposition of the omega matrix output from BayPass analysis placed most sites in the same region on PCs 1-3, with plots BxB_C, BxB_D and BxE_H more distant on all three axes (Fig. 4.3).

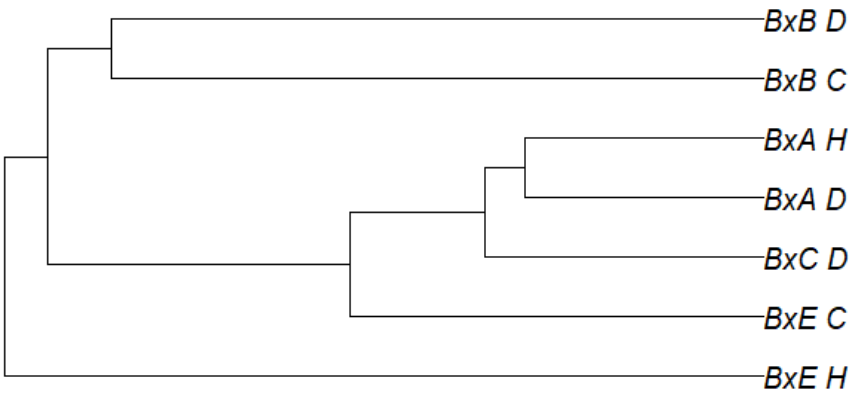

Figure 4.2 – Hierarchical clustering tree based on distance matrix derived from BayPass omega matrix $\hat{\Omega}$ (dij = 1-ρij). Branch length indicates relative similarity across all SNPs from hclust().

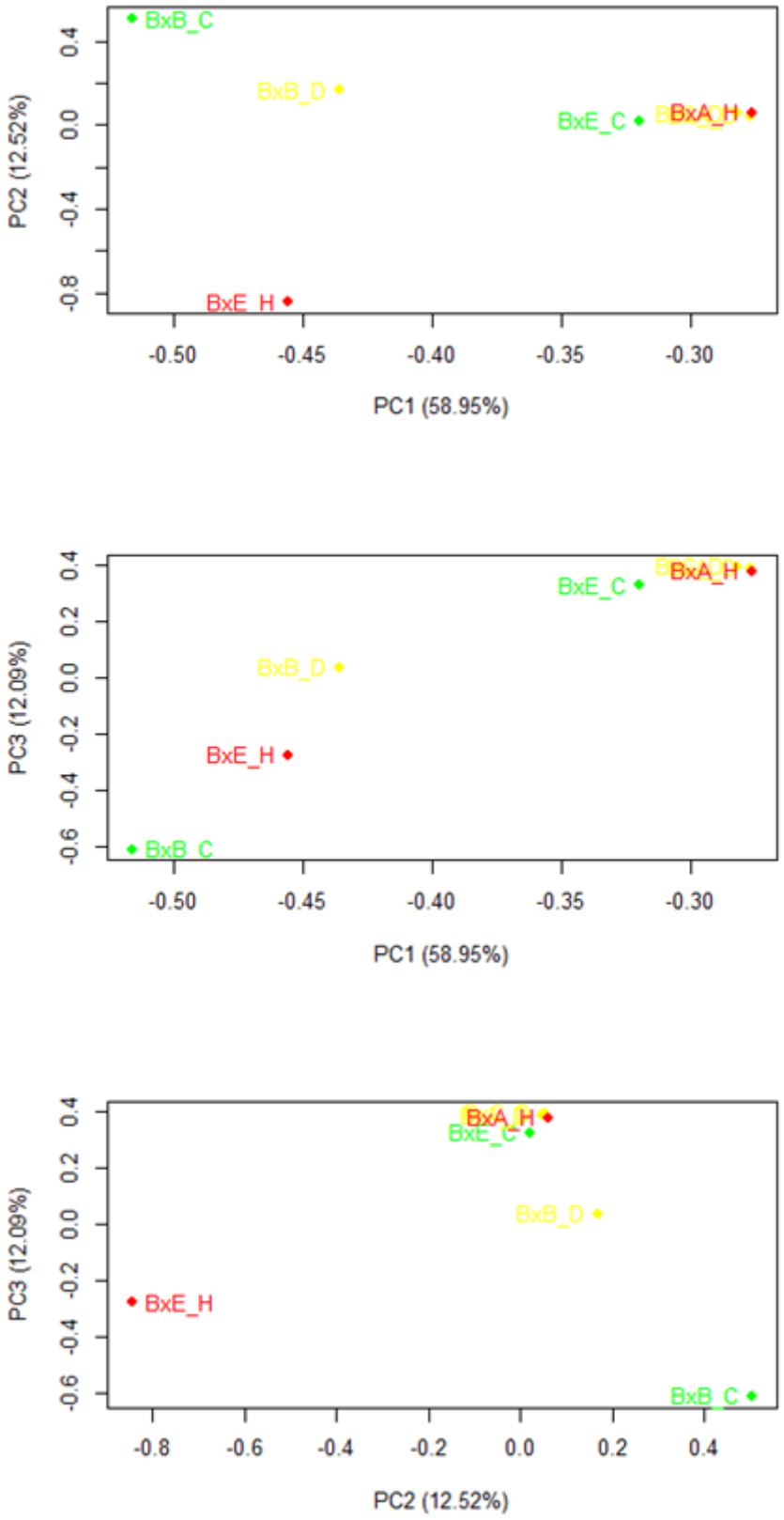

Figure 4.3 – Principal component analysis generated by singular value decomposition of the omega matrix output by the BayPass model, plotting PC's 1, 2 and 3 against each other.

4.4.1 Outlier SNPs

A total of twenty-one SNPs were identified as significantly differing between two or more treatments. Three SNPs were returned as outliers between control and drought treatments, compared with twelve for control vs heated and sixteen for heated vs. drought. Overlap between contrasts 2 (control vs. heated) and contrast 3 (drought vs. heated) consisted of seven SNPs, all with the relevant Bayes factor score above 20, giving high confidence that these SNPs are associated with the heated treatment. The overlap between contrast 1 (control vs. drought) and contrast 3 (drought vs heated) consisted of all three SNPs identified in contrast 1, all of which had Bayes factor scores that specifically attribute the variation to drought treatment. Mean F_{ST} for putatively neutral SNPs was 0.022 and 0.414 for those identified as outliers (ANOVA F = 357.7; P-value = < $2.2x10^{-16}$).

Of the twenty-one SNPs identified, eight were in genes (table 1), which are thought to have roles related to regulations of light control in development (*FEOV00050807*), regulation of transcription (*FEOV00045994*), protein folding and protective effects against physiological stress (*FEOV00039411*) and heat-stress tolerance (*FEOV00062933*). In addition is a trans-membranal protein of unknown function (*FEOV00012444*) and three proteins of unknown function (*FEOV00039224, FEOV00000561* and *FEOV00024168*).

Reducing the p-value threshold for contrasts to $10^{-4.5}$ revealed three additional SNPs, all in genes. The first of which (contig 615894, position 509) was in gene *FEOV00045981* and as associated with the heated treatment (contrast 2 p = $10^{-4.85}$; contrast 3 p = $10^{-4.75}$; Bayes factor 23.2). *FEOV00045981* is similar to *TYRAAT1*, an *Arabidopsis thaliana* chloroplastic gene that codes for an enzyme involved in tyrosine synthesis. The second (contig 623891, position 49344 – contrast 2 p = $10^{-4.97}$; Bayes factor 29.6) was four bases from another SNP (contig 623891, position 49340, see table 1), in the gene *FEOV00012444* for a transmembrane protein. The third was, again, associated with contrast 2 (p = $10^{-4.81}$; Bayes factor 40.4), situated in gene , with similarity to *RGA5*, a disease resistance gene in rice (*Oryza sativa japonica*).

Table 4.1 – Location and gene names, where applicable, for SNPs recovered as outliers. Contrasts refers to the contrast in which they were found to be significantly differentiated: C1 = control-drought; C2 = control-heated; C3 = drought-heated

SNP ID	Gene ID	Contig	Position	F_{ST}	Contrast
467		709	432725	0.368	C1, C3
5723		305,518	807	0.437	C3
5861		312,388	1938	0.789	C1, C3
5875		313,662	1548	0.314	C1, C3
7987		437,677	6375	0.887	C2, C3
10963		492,905	1963	0.307	C3
11565	*FEOV00045994*	511,341	10539	0.583	C2
12022		520,282	3534	0.371	C2
12235	*FEOV00039411*	522,429	12541	0.449	C2
14450	*FEOV00050807*	549,999	8911	0.361	C2, C3
15850	*FEOV00062933*	561,293	4038	0.357	C3
18548		585,882	14452	0.342	C2, C3
21666		606,424	716	0.338	C3
22648		612,559	1695	0.458	C2, C3
22651		612,559	1723	0.458	C2, C3
22652		612,559	1730	0.453	C2, C3
25632	*FEOV00012444*	623,891	49340	0.272	C2
28184		631,956	6874	0.213	C2
29726	*FEOV00039224*	636,383	4959	0.301	C3
38061	*FEOV00000561*	652,394	645650	0.356	C2, C3
40225	*FEOV00024168*	655,734	13858	0.284	C3

Notes on gene identity from the genome annotation:

FEOV00045994 - Similar to *MOT2*: General negative regulator of transcription subunit 4 (*Saccharomyces cerevisiae* (strain ATCC 204508 / S288c))

FEOV00039411 - Similar to Heat shock 70 kDa protein, mitochondrial (*Phaseolus vulgaris*)

FEOV00050807 - Similar to *FRS5*: Protein FAR1-RELATED SEQUENCE 5 (*Arabidopsis thaliana*)

FEOV00062933 - Similar to *HSFA2A*: Heat stress transcription factor A-2a (*Oryza sativa subsp. japonica*)

FEOV00012444 - Similar to *At3g47200*: UPF0481 protein *At3g47200* (*Arabidopsis thaliana*)

FEOV00039224, FEOV00000561 and *FEOV00024168* - Proteins of unknown function

GO terms (*Gene Ontology Resource*, n.d.) were available for four identified genes. *FEOV00045994* had terms for ubiquitin-protein transferase activity and the CCR4-NOT complex, a highly conserved domain in Eukaryotes involved in regulating protein function, gene expression, nuclease activity, and directing protein degradation and function. *FEOV00039411*, as a 70kDA HS protein was associated with ATP binding and

hydrolysis, protein folding and binding to unfolded proteins. A single GO term denoting regulation of DNA-templated transcription was attached to gene *FEOV00050807*. Finally, *FEOV00062933* was marked as having functions related to DNA-binding transcription factor activity, regulation of DNA-templated transcription and sequence-specific DNA binding.

The less significant genes *FEOV00045981* and *FEOV00069029* also had associated GO terms in the annotation. *FEOV00045981*, similar to *TYRAAT1* from *A. thaliana*, and differentiated in the heated treatment, simply described its function as an arogenate dehydrogenase, involved in tyrosine synthesis. The gene *FEOV00069029*, which likely codes for a fungal disease resistance protein, specifically similar to a protein involved in susceptibility to the common graminoid pathogen *Magnaporthe grisea*, is localised to the mitochondrial intermembrane space, and has ADP binding capabilities. Three of the flanking sequences for the unannotated loci returned hits via BLASTN, SNPs 12022, 18548 and 21666. 12022 aligned to a predicted EMS1 leucine-rich receptor kinase gene and 18548 to a SEC15A-like gene from cotton. 21666 aligned to several distinct possibilities, Hox-1, Rym4 and MCT-1.

4.4.2 Pairwise F_{ST} analyses

Pairwise F_{ST} calculations (table 4.2) for the individual populations, indicate a high degree of differentiation between most populations. Populations BxB_C, BxB_D and BxA_H had F_{ST} values above 0.15 compared to all other sites. BxA_D, BxC_D and BxA_H had low F_{ST} values relative to each other.

Table 4.2 – Pairwise F_{ST} values between all pools in analysis from poolfstat, asterisks (*) indicate an F_{ST} value above the significance cutoff of 0.150

BxE_C	0.226*					
BxA_D	0.218*	0.061				
BxB_D	0.291*	0.164*	0.155*			
BxC_D	0.225*	0.063	0.039	0.159*		
BxA_H	0.213*	0.062	0.033	0.155*	0.035	
BxE_H	0.330*	0.198*	0.188*	0.273*	0.197*	0.186*
	BxB_C	**BxE_C**	**BxA_D**	**BxB_D**	**BxC_D**	**BxA_H**

Outlier SNPs between control and drought plots had no differentiation for plots BxB_C and BxE_C, but the F_{ST} values for SNP 5861 was strongly differentiated between all drought plots (table 4.3). F_{ST} values between control and drought plots were strongly differentiated for all pairings of sites for SNP 5875, and for the majority (four out of six) for both 467 and 5861 (table 4.4).

Table 4.3 – Pairwise F_{ST} values for outlier SNPs between control and drought – within treatment comparisons, values above 0.15 marked with an asterisk

SNP ID	BxB_C-BxE_C	BxA_D-BxB_D	BxA_D-BxC_D	BxB_D-BxC_D
467	NA	0.373*	0.090	0.120
5861	0.002	0.989*	0.661*	0.234*
5875	0.097	0.074	0.016	0.002

Table 4.4 – Pairwise F_{ST} values for outlier SNPs between control and drought – between treatments comparisons, values above 0.15 marked with an asterisk

SNP ID	BxB_C-BxA_D	BxB_C-BxB_D	BxB_C-BxC_D	BxE_C-BxA_D	BxE_C-BxB_D	BxE_C-BxC_D
467	0.106	0.562*	0.258*	0.086	0.515*	0.233*
5861	NA	0.990*	0.673*	0.001	0.970*	0.625*
5875	0.399*	0.720*	0.583*	0.256*	0.533*	0.407*

Outlier SNPs between control and heated plots had significant (F_{ST} ≥0.15) differences between control plots and between heated plots. However, the values recorded between treatments were on average higher than for within treatment, with the exception of BxE_C-BxA_H (table 4.5). When visualised as a heatmap diagram (Fig. 4.4) it is clear that the F_{ST} values for SNPs 22648, 22651 and 22652 have some measure of linkage or covariance. Contrast 3, between heated and drought treatments, showed a clear difference in F_{ST} scores (tables 4.6 and 4.7) between the opposing treatments and the within-group measures, particularly for SNPs 38061,14450,15850, 10963, 467 and 29726 (Fig. 4.5).

Table 4.5 – Pairwise F_{ST} values for outlier SNPs between and within control and heated -between treatments in italics, values above 0.15 marked with an asterisk

SNP ID	BxB_C-BxE_C	*BxB_C-BxA_H*	*BxB_C-BxE_H*	*BxE_C-BxA_H*	*BxE_C-BxE_H*	BxA_H-BxE_H
7987	NA	*0.162**	*1**	*0.184**	*1**	0.856*
11565	0	*0.331**	*0.973**	*0.212**	*0.950**	0.552*
12022	0.038	*0.627**	*0.904**	*0.550**	*0.824**	0.079
12235	0.520*	*0*	*0.462**	*0.507**	*0.906**	0.248*
14450	0.019	*0.369**	*0.552**	*0.535**	*0.770**	0.120
18548	0.317*	*0.694**	*0.524**	*0.258**	*0.101*	0.033
22648	0.325*	*0.521**	*1**	*0.014*	*0.588**	0.517*
22651	0.326*	*0.521**	*1**	*0.013*	*0.587**	0.517*
22652	0.324*	*0.520**	*1**	*0.014*	*0.588**	0.517*
25632	NA	*0.401**	*0.368**	*0.344**	*0.329**	0
28184	NA	*0.344**	*0.409**	*0.221**	*0.254**	0
38061	0.478*	*0*	*0.199**	*0.493**	*0.141*	0.217*

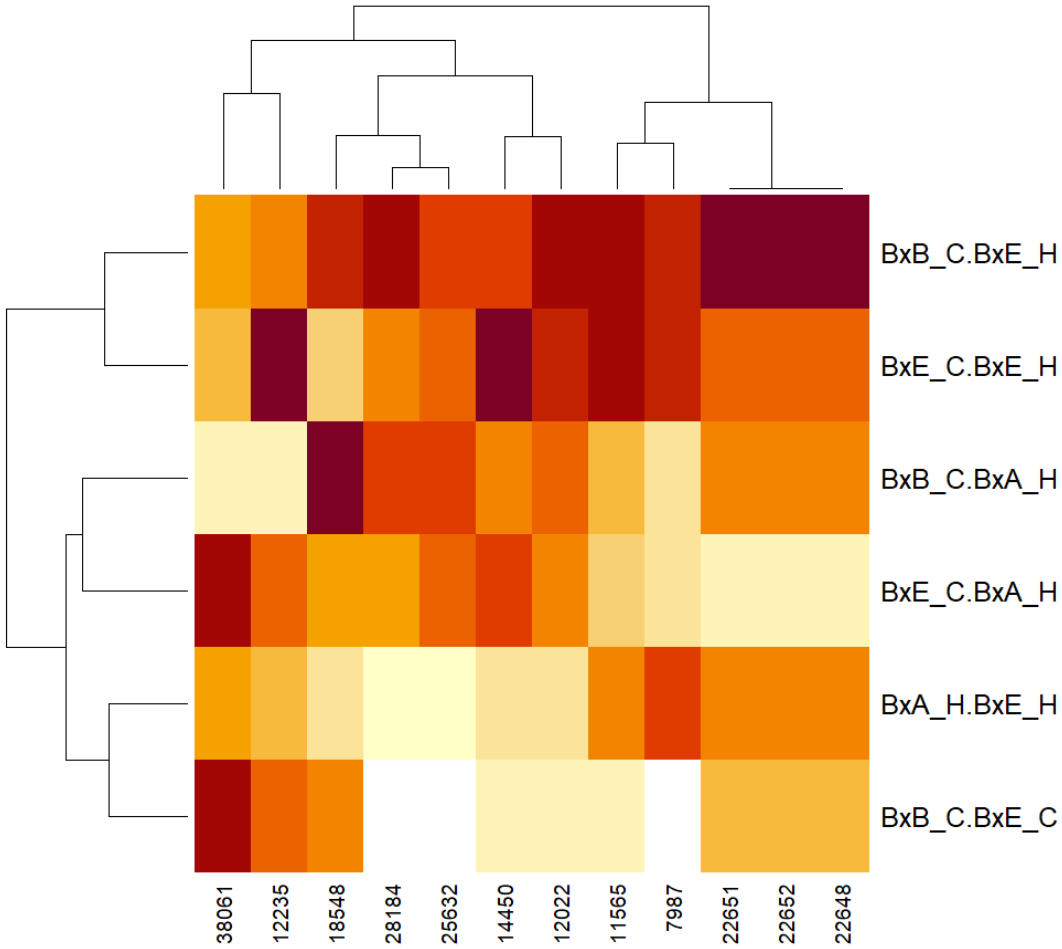

Figure 4.4 – Heatmap diagram of F_{ST} values displayed in table 4.5, lighter colours are lower F_{ST} values. As can be seen here, F_{ST} is slightly lower for most loci between control and control, and heated and heated, moreover, F_{ST} values for 22651, 22652 and 22648 appear to indicate genetic linkage. Trees are similarity using hclust(), *branch length indicating relative distance. Horizontal and vertical axes have been reordered in line with the* hclust() *ranking, grouping SNPs with similar patterns of allele frequency and paired populations with greater similarity.*

Table 4.6 – Pairwise F_{ST} values for outlier SNPs between heated and drought – within treatment comparisons, values above 0.15 marked with an asterisk

SNP ID	BxA_D-BxB_D	BxA_D-BxC_D	BxB_D-BxC_D	BxA_H-BxE_H
467	0.373*	0.090	0.120	NA
5723	0.207*	0.030	0.104	0.391*
5861	0.989*	0.661*	0.234*	NA
5875	0.074	0.016	0.002	0.240*
10963	0.131	0.227*	0.005	0.013
14450	0	0	0	0.120
15850	0.016	0.129	0.057	0.039
21666	0.130	0	0.142	0.285*
29726	NA	NA	NA	0.026
38061	0	0	0	0.217*
40225	0	0.030	0.005	0.388*

Table 4.7 – Pairwise F_{ST} values for outlier SNPs between heated and drought – between treatment comparisons, values above 0.15 marked with an asterisk

SNP ID	BxA_D-BxA_H	BxA_D-BxE_H	BxB_D-BxA_H	BxB_D-BxE_H	BxC_D-BxA_H	BxC_D-BxE_H
467	0.164*	0.165*	0.668*	0.670*	0.331*	0.332*
5723	0.158*	0.746*	0.461*	1*	0.295*	0.880*
5861	NA	NA	0.988*	0.992*	0.642*	0.725*
5875	0.073	0.452*	0.291*	0.782*	0.190*	0.652*
10963	0.093	0.131	0.386*	0.372*	0.465*	0.448*
14450	0.595*	0.825*	0.523*	0.711*	0.527*	0.778*
15850	0.180*	0.044	0.314*	0.150*	0.405*	0.283*
21666	0.268*	0.803*	0.020	0.391*	0.282*	0.808*
29726	0.330*	0.423*	0.392*	0.478*	0.275*	0.374*
38061	0.770*	0.406*	0.780*	0.429*	0.790*	0.396*
40225	0.134	0.667*	0.169*	0.818*	0.282*	0.821*

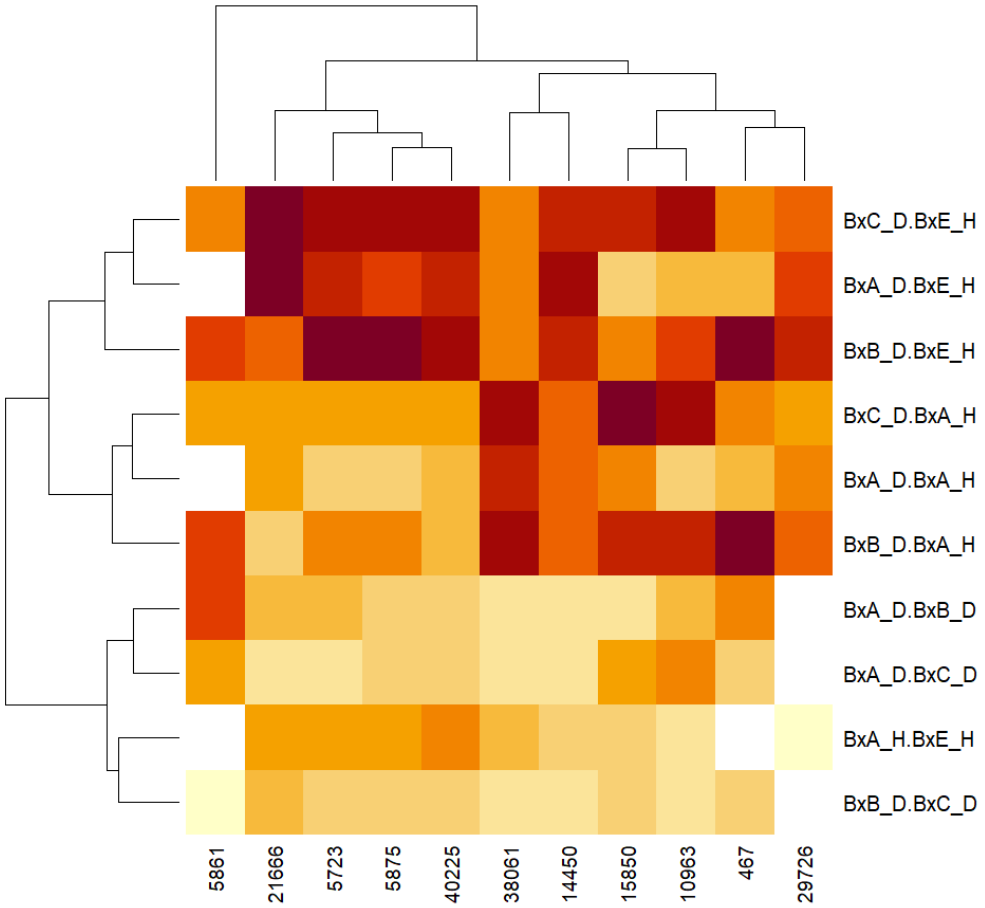

Figure 4.5 – Heatmap diagram of F_{ST} values displayed in tables 4.6 and 4.7, showing the lower F_{ST} between all four within-treatment comparisons, darker colours are greater F_{ST} values, usually indicative of stronger genetic structuring. Trees are similarity using *hclust()*, branch length indicating relative distance. Horizontal and vertical axes have been reordered in line with the *hclust()* ranking, grouping SNPs with similar patterns of allele frequency *and* paired populations with greater imilarity.

4.5 Discussion

The aim of this chapter was to investigate genetic changes seen in response to climate manipulation studies and compare these with those observed in naturally occurring microclimate populations. I sequenced a reduced representation pool of twenty-four individuals of *F. ovina* each from seven of the climate manipulation plots at the BCCIL, using Bayesian methods to identify loci under selection. In doing so I identified twenty-one SNPs differentiated between climate manipulations, identifying winter-warming treatment as the one with the largest degree of divergence. The loci under selection can be compared with those identified in the previous chapter, which used a similar approach with plants from eight north and ten south facing slope-aspect microclimate populations from the region surrounding the BCCIL. Of the identified SNPs, none were located in the same genetic regions as those identified for the wider Peak District site, however similar functions are inferred for some loci, and though representing different loci, both sets of populations had significant differentiation for genes similar to the disease resistance gene *RGA5*.

Loci identified with adaptation to the heated treatment had known functions associated with phenology, membrane structure, exocytosis and vesicle transport, pollen production and stress responses. *FEOV00030411* putatively codes for a chloroplastic 70kDa heat-shock protein, a ubiquitous family of chaperone proteins conserved across all domains of life, and important in tolerance of biotic and abiotic stress in plants (Aghaie & Tafreshi, 2020). Heat-shock proteins were named for their upregulation in *Drosophila* larvae after heat-shock, but have many roles in cellular function, regulating the expression and proper folding of proteins in all cellular compartments and are necessary for normal development and survival in plants. Mitochondrial members of the HSP70 family have confirmed roles in tolerance of heat stress in *A. thaliana* (Hu *et al.*, 2012) and rice (Sarkar *et al.*, 2013) as well as stress response in many other plant species (Rana *et al.*, 2018). *FEOV00050807*'s sequence suggests similarity to *FRS5*, a gene of *A. thaliana* involved in light-cycle mediated control of development and phenology (Ma & Li, 2018). A *FRS5* homologue in *Populus trichocarpa* was one of the genes associated with adaptation to differing daylengths in a large environmental-gene-association study (McKown *et al.*, 2014). It's possible that changes in the *FRS5* gene are related to alterations in annual growth cycles with increased winter temperatures.

FEOV00012444 has sequence similarity with *At3g47200*, a transmembranal protein with unknown function. It potentially has a *DUF247* domain, which may be linked to self-incompatibility loci in *Lolium* (Manzanares *et al.*, 2016). Though unconfirmed, this may tie in with two other identified loci: SNP 12022 which is similar to an *EMS1* gene in barley and SNP 18548 which aligns with *SEC15a* in cotton. Both of these genes are primarily associated with pollen development and microsporophyte function (Batystová *et al.*, 2022; Zhao *et al.*, 2002). The timing of the winter warming treatment is far removed from anthesis, leaving any link between these loci and response to climate selection unclear. However, their locations on different contigs and related functions imply some adaptive significance.

The contrast between control and drought treatments surprisingly only returned three loci under selection, none of which could be linked to gene functions explaining roles, though still statistically supported the differentiation. This is somewhat unexpected in the light of previous research identifying large numbers of loci differing between control and drought plots (Ravenscroft *et al.*, 2015). The contrast between drought and warming treatments though gave some possible adaptive significance. Genes and loci under selection between drought and heated treatments include the aforementioned SNP 18548 *SEC15a* and *FEOV00050807* as well as a *FEOV00062933*, related to the heat-stress-translation-factor-2a (*HSFA2a*) from rice. The Bayes factor for this gene associated it more strongly with the drought treatments than heat treatments. However, in rice studies, though other *HFSA* proteins are associated with drought stress, *HSFA2a* was exclusively associated with response to heat stress (Piveta *et al.*, 2020). Three of the genes strongly differentiated between heated and droughted plots had no known function.

Contrasting the loci identified in this study with those in the previous chapter identified no points of overlap. Functional similarities were seen though between genes seen in both though. *FEOV00069029* in the BCCIL samples was associated with the winter warming treatment and related to the disease resistance gene *RGA5*, *FEOV00017574* in the Peak District slope-aspect microclimate samples also codes for a gene similar to *RGA5*. BCCIL gene *FEOV00050807* and the Peaks gene *FEOV00058947* both code for transcription factors involved in regulation of development and flowering with light cycles and day length (Fornara *et al.*, 2009; Ma & Li, 2018; Sun *et al.*, 2015). More tenuously is the link between

the three loci identified at the BCCIL related to pollen formation and the evidence for divergence in an *Rf1*-like gene in the Peak district sites.

At the population level, hierarchical clustering analysis based on the results of the Bayesian analysis and pairwise F_{ST} across all SNPs came to similar conclusions regarding genetic structure. Pairwise F_{ST} recovered high levels of between-population differentiation with BxB_C as the most extreme outlier, followed by BxE_H and then BxB_D, with BxA_H, BxA_D, BxC_D and BxE_C very closely grouped. The Bayesian analysis recovered BxE_H as the most divergent population, grouping BxB_C and BxB_D together as more similar to each other than to the rest. Due to the small sizes of these pools (24 individuals per pool) it is possible that the relatively high F_{ST} values are an artefact of the small pool size and *F. ovina*'s high within-population variation. As in the work of Bengtsson *et al.* (2004), due to the outbreeding nature of *F. ovina* and the stochastic effects of pollen clouds involved in pollination, variation in allele frequencies can be unexpectedly high at small (<11m) spatial scales when compared with sampling more distant (>20km) populations.

Alternatively, Trinder *et al.* (2019) established that there were changes to flowering phenology and a level of assortative mating between *F. ovina* from the drought and control plots at the BCCIL. These effects could drive rapid divergence between plots, leading to elevated F_{ST} values. The extreme divergence of BxB_C, a control plot, is hard to rationalise, and may prove to be a sequencing error, however the physical separation between the two heated plots sequenced, one at the top of the site and the other at the very bottom (see figure 4.1), at least has the potential to be a result of limited gene-flow.

A methodological error in the design of some of the GBS adaptors led to the loss of a number of the samples in this study, which was not recognised until demultiplexing. As a result, no data could be included for the watering treatment and only two to three each of the other treatment pools had enough sequences to generate reliable allelic frequency data. A prior study using AFLP data indicates that the effect of experimental block is insignificant for *F. ovina*, though that could not be confirmed in this study it supports the findings (Ravenscroft *et al.*, 2015).

The data presented here broadly agrees with the results of prior studies, finding that the *F. ovina* population at the BCCIL has undergone adaptive evolution to climate in the 25 years

since the experiment was established. Furthermore, though SNPs identified did not exactly match those derived from the slope-aspect microclimate study in the wider Peak District, the putatively similar functions at several prominent loci lend support the hypothesis that data from the BCCIL is comparable with the processes occurring under natural conditions and suitable for extrapolation to wider systems. As a final conclusion, the magnitude of selection, in light of the site's native heterogeneity, may indicate that this rapid evolutionary change may represent an example of microclimate's ability to buffer populations in response to sudden shifts in climate by maintaining adaptive genotypes within the larger population. Further avenues for study might include a similar, pooled GBS approach using populations from the BCCIL and a selection of nearby slope aspects to search for evidence of introgressive gene flow.

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5 The effect of commercial propagation on allele frequency in *Festuca ovina*

5.1 Abstract

Selection of source material is an important step in conservation interventions involving the reseeding of degraded habitat, particularly in the face of a changing climate (Breed *et al.*, 2013; Schröder, 2008; Thomas *et al.*, 2014). Populations of plants are commonly locally adapted to their environment, indicating that environmental factors strongly influence fitness and survival in plants. It follows then that in any conservation intervention centred around the introduction of plants to a location should be approached with the adaptive potential of the material in mind. A key challenge in large-scale reseeding interventions is the acquisition of sufficient seed to establish a self-sustaining population, and to ensure that seed contains the relevant genotypes to thrive at the site in question as hand collection of seed is impractical above a certain scale. Emorsgate Seeds is a company, based in Norfolk, UK, that specialises in the collection and propagation of several hundred species of wild plant seeds for use in conservation efforts. Amongst the species they offer is *F. ovina*, representing six generations of cultivation since initial collection from the wild, which is often sown in bulk on projects aimed at stabilising bare soil and creating species rich grasslands. In order to understand the selective effects of mass propagation, I applied genotyping-by-sequencing to pools of samples from their fourth and sixth generation of plants, and material from the original collection site for comparison. I observed no significant differences in allele frequency, nor loss of alleles, associated with cultivation, out of a collection of over 72 thousand SNPS. These results are consistent with literature regarding the allele frequency of commercial varieties compared with wild populations, and the tendency for self-incompatible, wind pollinated species like *F. ovina* to retain genetic diversity.

5.2 Introduction

Restoration ecology is the conservation science of restoring degraded habitats and ecosystems. Habitat destruction, fragmentation and land use change are important causes of habitat degradation, with modern scholarship also focusing extensively on climate change

as a driver (Coleman *et al.*, 2020; Hodgson *et al.*, 2005; Sork & Smouse, 2006). One of the principal elements of restoration interventions for plants has been reseeding, the purposeful addition of propagules to an area in order to (re)introduce or bolster extant plant communities or populations (Maschinski & Haskins, 2012; McKay *et al.*, 2005; Neale, 2012). However, concerns have been raised about the suitability of certain seed-sourcing strategies used, due to predicted climate change (Breed *et al.*, 2013). Improper selection of propagule material has the potential to increase risk of failure through introduction of poorly adapted or maladaptive genotypes. As restoration interventions had an estimated global cost of £3.5 trillion per year (adjusted to 2022 monetary value) in 2008 (Williams *et al.*, 2014), it is important that interventions are undertaken with the greatest chance of success.

The success of plant introduction is dependent on adaptive features of the source population and target site, as plants commonly show local adaptation to climate, amongst other factors. Reciprocally transplanted *Arabidopsis thaliana* populations between Sweden and Italy had significant differences in survivorship and fecundity for population x site, demonstrating clear home-field advantage over two of three winters (Ågren & Schemske, 2012). Seedlings of the neotropical live oak *Quercus oleoides* collected from eight populations (81 maternal families) across Central America, grown in a common garden demonstrated variation in functional traits, such as specific leaf area (SLA) and leaf-nitrogen content related to climate of maternal origin (Ramírez-Valiente *et al.*, 2017). The potential for these differences to influence successful establishment is demonstrated in the 2020 study by Zirbel and Brudvig (2020): Seventy species, studied across twelve prairie restorations at W.K. Kellogg Biological Station in Michigan, found plant functional traits to be predictive of successful establishment in a reseeding experiment. Root traits influenced survivorship and establishment with regard to soil moisture content, SLA with light intensity and leaf nitrogen content with herbivory (Zirbel & Brudvig, 2020).

The idea of “local is best” is prevalent in restoration circles, supported by the body of evidence for local adaptation’s influence on plant population dynamics and fitness. However, what constitutes “local” and the universality of this assumption are subject to question. A study of five graminoids (*Agrostis mertensii, Avenella flexuosa, Carex bigelowii, Festuca ovina* and *Poa alpina*) and one hawkbit (*Scorzoneroides autumnalis*) across Norway

found four general regions of seed transfer (Jørgensen *et al.*, 2016). For three of the species (*F. ovina*, *A. flexuosa* and *S. autumnalis*) there was little meaningful differentiation across the whole of Norway, suggesting that for these species, "local" populations may cover enormous areas. The importance of using local provenances is predicated partially on the well supported notion that populations are locally adapted to their environments. However, as the climate continues to change, local populations may become maladapted to local conditions, meaning that strict adherence to local seed sources has the potential to set restoration initiatives up for future failure (Prober *et al.*, 2015).

Several approaches to the sourcing of seed for restoration have been proposed over the last two decades, with the aim of generating robust, self-sustaining populations. Composite provenancing is a variation of local provenancing in which precedence is given to local genotypes, with progressively lower contributions from more distant populations, aiming to preserve genetic character while ensuring adaptive potential (Broadhurst *et al.*, 2008). Predictive provenancing involves sourcing propagules from populations growing under conditions thought to be representative of future conditions at a target location. Broader admixture has been suggested, using many sources in equal quantities with the aim of generating novel combinations of genes, creating a population with strong adaptive potential – novel genotypes for novel conditions (Breed *et al.*, 2013). Most recently, Prober *et al.* have suggested an approach they call "climate adjusted provenancing", a modified combination of admixture and predictive provenancing, using genotypes collected from along a climate gradient, weighted towards the predicted future conditions (Prober *et al.*, 2015). However, the collection of seed from wild populations is not practical for direct sowing in large-scale seeding projects. These projects require a scaling up of seed, usually through several generations of cultivation, in order to generate sufficient quantities for use, raising the question of whether this genetic variation can be maintained through the propagation stage.

Emorsgate Seeds is a company based in King's Lynn, Norfolk who specialise in the propagation of wild, native plant seeds for restoration work and landscaping. Established in 1980 to provide seed for grassland restoration, based on the work of Dr T. Wells at the Institute for Terrestrial Ecology at Monks Wood, they now provide over 200 species for reseeding projects and applications. With 42 years' experience of collecting and propagating

native plant seeds at their sites in Norfolk and Bath, Somerset (Wildseed.co.uk, 2022) they have been involved in supplying seeds to numerous restoration projects including the Devil's Dyke in Sussex and the Baldock Bypass in Hertfordshire.

The company has been actively engaged with scientific research to support and inform their practice. A study conducted at the King's Lynn site using water traps assessed the floral attractiveness of non-crop vegetation on insects for 13 plant species, with regard to their benefit in field margin planting (Carrié *et al.*, 2012). Nichols, Goulson and Holland worked with Emorsgate to identify 14 wildflower species most utilised by wild bees at the site in Norfolk, noting that the majority of which were not found in government recommended mixes (Nichols *et al.*, 2019). Further work by the same authors, in association with Emorsgate, trialled seed mixes, finding that inclusion of meadow and cornfield annuals improved establishment of floristically rich, perennial plant communities (Nichols *et al.*, 2022). As part of their commitment to evidence-based practice they partnered with my project to investigate the effects of their propagation strategy on the climatically adaptive potential of their *F. ovina* stocks.

The propagation strategy employed at Emorsgate Seeds begins with a manual collection of seed from a specific site. In grassland habitats at least, collection takes the form of a zig-zag or random walk through the site collecting mature seed heads from a variable number of maternal plants. Wild collected seed is sown in seed trays or pots in a polytunnel at one of their locations and allowed to develop to reproductive maturity and set seed. Depending on quantities of seed produced in this step, the next generation may be planted out to small plots for a further generation, or straight to tilled fields. Once sufficient plants have been generated, harvesting of seed for sale can begin. Fields are planted in rows and, where possible, harvested with standard agricultural machinery.

Evidence has been found for agricultural propagation of wild plant seeds exerting selection pressures on the populations, as well as evidence for retention of genetic variation. There is precedent for the accidental selection for seeding traits, such as larger size, greater fecundity and uniformity of seed maturation in grasses grown and harvested in an agricultural setting: namely the domestication of our major grain crops (Hillman & Davies, 1990; Purugganan & Fuller, 2009). Dyer and Knapp found evidence that harvest time in commercially propagated populations of the grass *Nassella pulchra* selected strongly for

early or late maturing genotypes (Dyer *et al.*, 2016). *Clarkia pulchella* plants after eight generation of commercial propagation performed poorly in a greenhouse-based growth test in terms of overall survival, drought resistance, excessive water resistance, flowering rate and relative fitness compared to plants grown from seed collected from two of the three progenitor populations. The authors of the study attributed this to accidental selection and/or population bottlenecks as a result of collection and cultivation (Pizza *et al.*, 2021). Conversely, a study on five species (*Achillea millefolium*, *Centaurea cyanus*, *Galium album*, *Medicago lupulina* and *Plantago lanceolata*) cultivated by a German supplier of native plant seeds over five generations only found evidence for evolutionary change in the selfing *M. lupulina*, but none for the other four, outcrossing species (Nagel *et al.*, 2019). Comparison between commercial populations, wild and *ex situ* populations of the prairie sunflower *Helianthus maximiliani* found differences between commercial populations and the others. However, it was also found that commercial populations retained a greater number of rare-alleles relative to the others (Braasch *et al.*, 2021). Combining these findings with the fact that *N. pulchra* and *C. pulchella* are both known to engage in selfing behaviour suggests that risk of large genetic changes in commercial populations may be influenced by mating system, and must be assessed on a species-by-species basis.

Emorsgate Seeds' current stock of *F. ovina* is on its sixth generation in cultivation since collection from the wild at a site called Grass Wood, Yorkshire, in 2006. Diploid populations of *F. ovina* are self-incompatible, while reports on tetraploids range from self-incompatible to "moderately" self-fertile (Harberd, 1962; Watson, 1958). Due to its largely outbreeding and wind-pollinated nature, *F. ovina* may be expected to maintain genetic variation better under cultivation compared with selfing plants (Berge *et al.*, 1998). However, this does not preclude the possibility of accidental selection, particularly for traits related to seed set, along with bottleneck/founder effects arising from collection, and early in the collection and propagation process. The initial wild collection of seed was made on a single day, limiting it to plants in the population with ripe seed at that time and, as it was collected as seed heads, many seeds belonged to the same maternal half-sib families. The cultivation site is also somewhat atypical for *F. ovina*, with deep soil and ample moisture — *F.* ovina is typically a plant of "challenging" habitats and thin soils. Considering the established importance of maintaining or fostering the adaptive potential of populations in restoration projects, there

is a need to understand the effects cultivation may have on allele frequencies in climatically adaptive genes.

This chapter aims to address the question of whether commercial propagation of *F. ovina* leads to changes in the allele frequency at loci associated with climatic adaptation, using seed from three generations from Emorsgate Seeds population, and material collected from the original collection site at Grass Wood in Yorkshire. To do this, I applied GBS to pools from the fourth, fifth and sixth generations and the Grass Wood samples, in the same manner as the BCCIL and Peak District samples. Building upon the work in chapters 3 and 4, that identified loci associated with climatic conditions, I investigated two key questions: whether the cultivated populations differed significantly from the parent field population, and the identity of any loci found to be under selection, either for climate or propagation systems. The results of this study found no statistically significant evidence for either loss of alleles nor evidence of alleles under selection with regard to cultivation.

5.3 Methods

5.3.1 Sample collection

Samples were collected from four populations of *F. ovina*, three generations of seed provided by Emorsgate Seeds and a collection made from the original collection location at Grass Wood (OS six-figure grid reference: SD983652) near Grassington in Yorkshire. The Grass Wood pool (hereafter: GW) consisted of 94 samples of vegetative tissue, stored in silica gel immediately (Chase & Hills, 1991). The site did not allow for grid-based sampling, nor was the original sampled in that manner. Sampling had to be done on an ad-hoc basis, ensuring that tillers came from distinct tussocks and no tussock was sampled twice until the full number of samples had been collected.

Four generations of seed provided by Emorsgate were sown, 3rd, 4th, 5th and 6th (hereafter: Emg3, Emg4, Emg5 and Emg6 respectively) in square pots of John Innes No. 1 compost and grown in a grow-room at the University of Liverpool Biosciences building for 4 weeks, until large enough to harvest. Germination was good for Emg4, Emg5 and Emg6, however the seed from Emg3 failed to germinate and was excluded from the study. Once large enough to harvest, seedlings were sampled one-by-one starting from a corner of the pot until 94 had been harvested of each generation. To ensure no bias, plants were collected uniformly,

moving out from the initial corner, regardless of size, though no obvious discrepancy in growth rate was noted between generations or individuals. Samples were immediately placed in silica gel to dry.

5.3.2 DNA extraction, library preparation and sequence processing

DNA extraction, pooling, sample handling and library preparation was handled as in chapter 3. DNA was extracted using a modification of the method described in Werth *et al.* (2016), pooled in equimolar concentrations and digested with the enzymes EcoRI-HF and HhaI-HF (NEB) and ligated to barcoded adaptors for amplification. Amplification was done using standard Illumina indexing primers for the HiSeq4000 platform, fragments were size selected for sizes between 300 and 700bp, and sequenced using S4 chemistry.

Sequence processing followed the same protocol outlined in chapter 3: Samples were demultiplexed using CutAdapt (Martin, 2011), and aligned to a draft genome compiled by Dr Raj Whitlock using the program hisat2 (Kim *et al.*, 2019). Alignments for the four samples (Emg4-6 and GW) were filtered to exclude repetitive regions using BedTools Intersect (Quinlan & Hall, 2010), merged into a single BAM file using Samtools (Danecek *et al.*, 2021) and variants called using FreeBayes (Garrison & Marth, 2012). The sample Emg5 sequenced poorly and had to be discarded due to high levels of missing data. The final VCF consisted of 72,413 SNPs with a minimum read depth of 100 per sample and no missing data.

5.3.3 Statistical analysis

Data from the VCF file was extracted to pooldata format using the R (R Core Team, 2020) package poolfstat (Gautier *et al.*, 2022). The same R package was used to generate pairwise and total F_{ST} values (functions *computePairwiseFST()* and *computeFST()*, with the method "anova"). Analysis was performed in BayPass 2.3 (Gautier *et al.*, 2013) was run using the covariate model, coding location, i.e. Grass Wood vs. Emorsgate's site in Norfolk, as opposing environmental variables. Contrasts were set at four levels: Emg4 vs Emg6; Emg4 vs GW; Emg6 vs GW; Emg4 & Emg6 vs GW. Outlier SNPs were identified as those with Bayes factor ≥ 20, p-value of ≤ 0.00001, and $F_{ST} \geq 0.15$, as reported by the BayPass and poolfstat. Outliers were extracted to a new VCF file and annotated using a GFF file provided by Dr Raj Whitlock.

5.4 Results

Analysis of the allele frequency of biallelic SNPs between the three populations failed to find any clear outliers as defined in section 5.3.3 (Fig. 5.4). A single SNP on contig 598786, position 4676, had a Bayes factor over 20, but a p-value of 0.724 and an F_{ST} of 0.075. No SNP locus has a p-value below the threshold value of 0.00001 derived from the contrast model or for the XtX statistics generated between all populations.

A singular value matrix based principal component analysis of omega (Fig. 5.3) resolved Emg4 and Emg6 as closer together on PC1 (50.31% of variance explained), with Emg6 closer to GW on PC2 (28.97% of variance) and Emg4 to GW on PC3 (20.71% of variance). A correlation matrix generated from omega using the R function *cov2cor()* found correlation was higher between the two Emorsgate samples than between them and Grass Wood (Fig. 5.1), and lower between the sixth generation sample and Grass Wood than between the fourth generation and Grass Wood. The distance matrix (Fig. 5.2) was used to generate a tree, recovering Emg4 and Emg6 as closer to each other than Grasswood. Pairwise F_{ST} values indicate high levels of differentiation between all three populations, ranging from 0.461 between Emg4 and Emg6 to 0.538 between Emg4 and GW (table 1). Though no outliers were identified by Bayes factor or contrast analysis, summary analysis of the pairwise F_{ST} values generated from *poolfstat* identified 3,366 SNPs in which F_{ST} between Emg4 and Emg6 was less than 0.15 but greater than, or equal to, it for both Emg4-GW and Emg6-GW. However, identifying SNPs where Emg4-Emg6 and Emg6-GW were differentiated but Emg4-GW was not recovered 1,985 SNPs; a further 2,383 SNPs were differentiated between Emg4 and Emg6, and Emg4 and GW, but not between Emg6 and GW. These values, drawn from the 46,520 SNPs for which there were no NA values for pairwise F_{ST}, represent 7.2%, 4.2% and 5.1% of the total. Though the specific allelic frequencies differed between samples, overall genetic diversity for pools remained largely similar between the cultivated generations and the wild population. Figure 5.5 displays a density plot of allelic frequencies for SNPs, comparison of allele frequencies between GW, Emg4 and Emg6 found no loci at which any alleles had been lost from the cultivated populations.

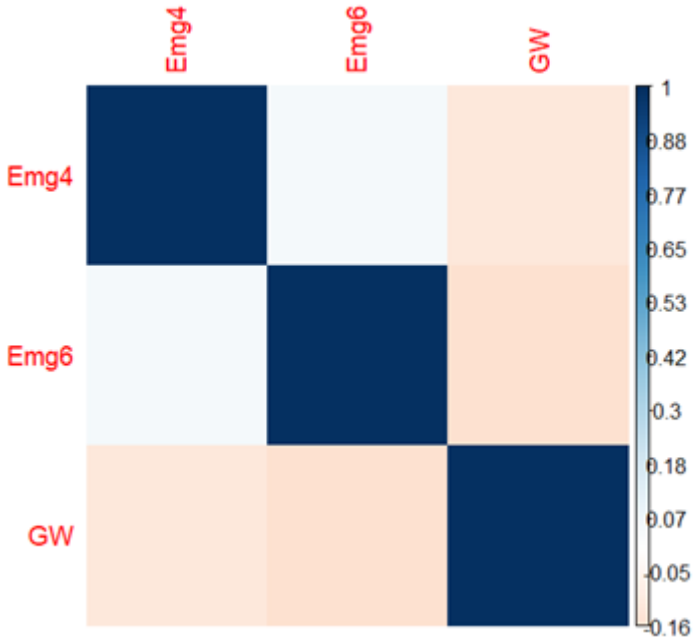

Table 5.1 – Pairwise F_{ST} values for all three populations generated from poolfstat

	Emg4	**Emg6**
Emg6	0.461	NA
GW	0.538	0.436

Figure 5.1 – Plot of correlation matrix derived from omega covariance matrix. Correlation is higher between Emg4-Emg6 (value = 0.049) than between Emg4-GW (value = -0.123) or Emg6-GW (value = -0.164)

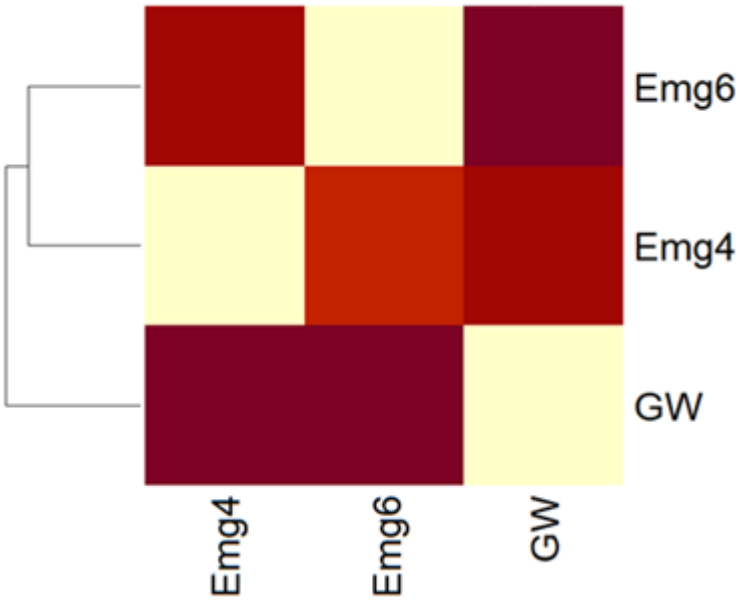

Figure 5.2 – Heatmap of $\hat{\Omega}$ om (dij = 1-ρij), distance matrix generated from BayPass omega matrix, lighter colours are greater similarity, tree on righthand side shows grouping by genetic similarity. Trees are similarity using hclust(), *branch length indicates relative distance in the dissimilarity matrix.*

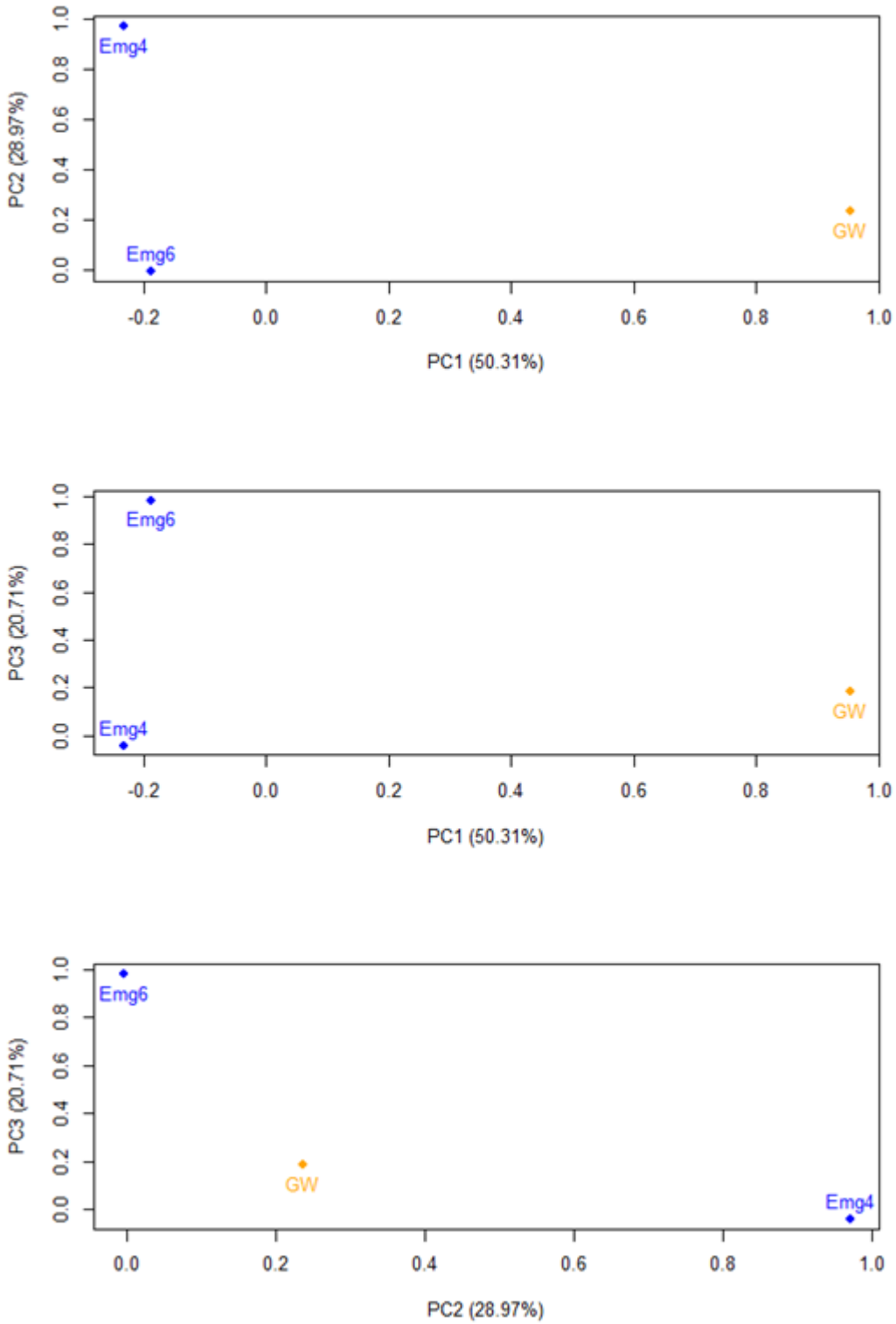

Figure 5.3 - Principal component analysis generated by singular value decomposition of the omega matrix output by the BayPass model. Though PC2 and PC3 (explaining 28.97% and 20.71% of the variation respectively) recover Grasswood as closer to Emg6 and Emg4 respectively, PC1 (50.31% of variation) recovers Emg4 and Emg6 as much closer to each other than to Grasswood.

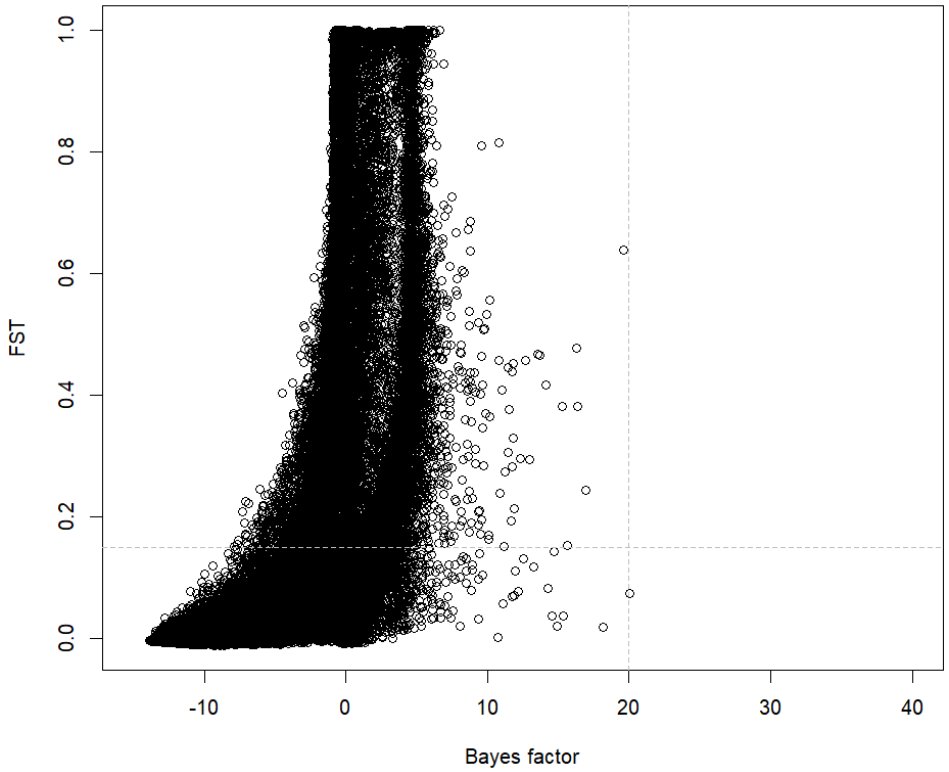

Figure 5.4 – Plot of Bayes factor against F_{ST} for al SNPs. Vertical line marks the BF=20 significance threshold, horizontal line marks the F_{ST}=0.15 significance threshold. As can be seen here, no single SNP met both criteria for significance, with only one having a Bayes factor ≥ 20, despite many having a high F_{ST} value.

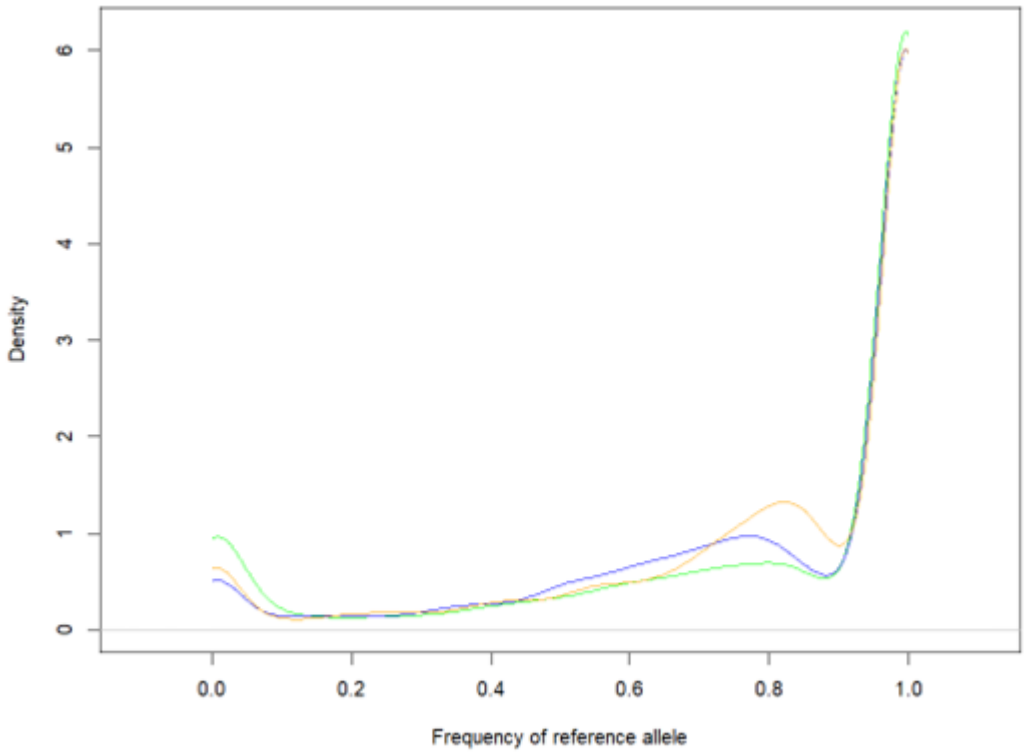

Figure 5.5 – Density plot of posterior allele frequencies for the reference allele (i.e. the allele found in the reference genome). Though there is some change in allele frequencies in the 0.5-0.8 range, overall patterns remain similar. Of note though is the higher level of 1.0 and 0-0.02 seen in Emg4, suggesting a higher percentage of homozygotes. Emg4 = green, Emg6 = orange, GW = blue

5.5 Discussion

The aim of this study was to identify loci under selection, i.e. signs of early domestication, in *F. ovina* populations being cultivated by Emorsgate Seeds, to assess the risks of commercial propagation to genetic diversity. The ultimate finding of this chapter was that there were no loci under selection, nor any alleles even lost from the population, from a total number of over seventy-two thousand SNPs. These results may not be altogether surprising, the mating system of *F. ovina* – highly outbreeding, self-incompatible, wind pollinated – is known to lend itself to the maintenance of genetic diversity within a population, and resisting inbreeding (Berge *et al.*, 1998). Previous studies investigating the effects of active, intentional selection on *F. ovina* likewise turned up high levels of genetic variation and allelic diversity retained in cultivated population (Weibull *et al.*, 1991).

High between-population F_{ST} values appears to be driven by generation-on-generation differences in allele frequency, however, these changes are not consistent between generations four and six, indicating random effects rather than directional selection. The growth site at Emorsgate Seeds is flat and open, a mesic habitat, and simply may not be exerting selection pressures strong enough to differentiate populations over the course of six generations. As the starting population consisted of multiple seed-heads from metres apart, starting variation was likely high and population sizes from the first generation onwards large enough to avoid excessive drift. However, as seed was collected from a single population, and no major changes are observed in alleles present, per results from chapters 3 and 4, it's possible the source population is locally adapted to its conditions – lowland, relatively flat and shaded by adjacent woodland – and may lack relevant variation for other climatic conditions.

A point of confusion arises from the pairwise F_{ST} values generated by *poolfstat*, which show high F_{ST} values between all populations. Though Bayesian analysis recovers the two cultivated generations included in the study as closer related (as would be expected), F_{ST} calculations recovered the sixth generation and Grasswood as less divergent to each other than they were to the fourth generation. However, BayPass recovered the more intuitive finding that Emg4 and Emg6 were closer to each other than they were to Grasswood (see Fig. 5.3). It is hard to reconcile the F_{ST} values with the known history of the populations and

the findings of the BayPass analysis, and with the F_{ST} values recovered for the samples from the Peak District in chapter 3, with the most parsimonious explanation at this point being that the samples themselves were poorly handled or sequenced. As such, I feel that, without a repeat of the experiment to confirm these counter-intuitive results, little can be reliably drawn from them.

One other alternate explanation for the unexpected F_{ST} values exists: seed age and viability. Four generations of seed were sown (3rd, 4th, 5th and 6th), which were harvested in different years. Notably, as described in the methods section, the oldest seed (3rd generation) failed to germinate entirely, therefore it is possible that the plants grown from fourth generation seed have undergone a post-harvest selective sweep. *F. ovina* seeds generally germinate within a couple of months of ripening, with the autumn rains, and are not particularly well adapted for long-term survival in the soil seed bank. Though there appears to be no research on this in *F. ovina*, studies on *Brassica napa* (Gruber *et al.,* 2004) and *Trifolium subterraneum* (Evans & Smith, 1999) found genotype affected soil seedbank persistence. Furthermore, studies comparing growing populations and seed bank populations of the plants *Lesquerella fendleri* (Cabin *et al.,* 1998) and *Plantago lanceolata* (Tonsor *et al.,* 1993) found differences in genotypes present. If we consider human storage as potentially analogous to seed bank persistence, it's not impossible that the difference in F_{ST} seen between Emg6 (short harvest-germination interval), Emg4 (longer harvest-germination interval) and GW (material directly collected from wild population) are a result of differential rates of attrition of genotypes in storage.

In conclusion, most of the results of this study seem weak, and I would be hesitant to claim reliable conclusions can be drawn regarding allele frequencies from these populations. However, the lack of any alleles from biallelic loci being lost between the Grasswood samples and the two Emorsgate generations included in the final analysis may still be used to infer that genetic diversity is being maintained within the cultivated population at present.

5.6 Bibliography

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6 General discussion and synthesis

The major findings of this thesis may summed-up in terms of the four data chapters presented within. Firstly, chapters 2 and 3 provide strong evidence for evolutionary change in *Festuca ovina* populations in the Peak District associated with microclimatic variation, visible in morphological phenotype and genotypic information. Secondly, based on the data presented in chapters 3 and 4, the evidence for rapid evolution in response to climate seen in *F. ovina* at the Buxton Climate Change Impacts Laboratory (BCCIL) appears parallel in nature to that seen in old, naturally occurring microclimate populations. Directly comparable alterations in gene function were seen between heated and droughted treatments and differences seen between north and south facing slope aspects. And thirdly, from chapter 5, despite 6 generations of cultivation at their site in Norfolk, no evidence is found for allele loss at any loci amongst Emorsgate Seeds' stocks of *F. ovina*. Their cultivated stock retains levels of genetic diversity comparable with the wild progenitor population, and is presumed to retain any climatically adaptive genetic variation present in the source population.

6.1 Genetic variability and population structure in *F. ovina*

Climate change exposes plants to increased levels of stress that locally adapted populations may be ill-equipped to deal with (Gitlin *et al.*, 2006; Jump & Peñuelas, 2005; Román-Palacios & Wiens, 2020). Local adaptation in plants is well known from classic reciprocal transplant experiments that demonstrate the deleterious effects of non-local environmental conditions on plant survival and performance (Ågren & Schemske, 2012; Macel *et al.*, 2007), an effect that is expected to be seen in plants exposed to rapid climate change. However, certain types of local adaptation may have a protective effect for plant populations exposed to climate change – local adaptation associated with microclimate sites in a heterogeneous landscape has the potential to act as reservoirs of pre-adapted genotypes (De Kort *et al.*, 2020) that can be expected to buffer populations against these effects (Ahrens *et al.*, 2019; Razgour *et al.*, 2019). So long as appropriate genotypes are present, plant populations can adapt to extreme climatic shifts as selective sweeps increase the frequency of climatically adaptive variants within the population (Jump *et al.*, 2008). Vital to this capability though is

the presence of standing variation within populations, that allow for adaptation to these new selective pressures.

Festuca ovina is an outbreeding, self-incompatible, wind pollinated plant with a relatively short flowering period known to show high levels of genetic variability within populations (Berge *et al.*, 1998; Watson, 1958). This mating systems favours the retention of genetic variants within a population unless strongly selected against, with the effect of minimising between-population genetic structure at selectively neutral loci (Berge *et al.*, 1998). Studies on patterns of allelic divergence between populations in Fennoscandia found genetic variance to be high between samples collected at very small (within population, distances of under 11 metres) and very large (hundreds to thousands of kilometres distant) scales. But, at intermediate scales between-population comparison tended to show less pronounced genetic structure (Bengtsson *et al.*, 2004). This pattern of extensive within-population variation can be seen in samples from the Peak District and BCCIL populations presented in this thesis, in which spatially and climatically separated populations mostly have low overall F_{ST} values across thousands of selectively neutral loci. This pattern however is not seen in the Emorsgate Seeds population, which demonstrated uncharacteristically high between-generational F_{ST} values, indicating significant genetic divergence, however, no significant loss of allele diversity was detected.

Habitat heterogeneity structured around varied microclimatic patches has the potential to buffer populations against effects of rapid climatic change while fostering and maintaining genetic diversity at climatically adaptive loci (Denney *et al.*, 2020; Lampei *et al.*, 2019; Oldfather *et al.*, 2020; Oldfather & Ackerly, 2019; Olliff-Yang & Ackerly, 2020; Prentice *et al.*, 1995). Together the phenotypic data from chapter 2 with the genotypic data from chapter 3 indicate that slope aspect microclimate sites drive the formation and maintenance of differentially climatically adaptive genotypes for a range of conditions within the larger metapopulation for *F. ovina* populations in the Peak District.

A minor possible correlation between the data from chapters 2 and 3 are in the outlier sites. Site S001, located at Mill Dale, stood out as one of the most divergent sites in terms of genetic identity, as one of the four sites with high F_{ST} values separating it from the majority of others (Ch. 3 table 3). In terms of morphology, individuals from site S001 were the shortest (Ch. 2 fig. 7), had the greatest number of tillers (Ch. 2 fig. 6) and the greatest

specific leaf area. Site S002 was atypical in morphology relative to other south facing slopes, it was the tallest south facing (Ch. 2 fig. 7), with the fewest number of tillers of any site (ch. 2 fig. 6) and in the genetic analysis and had F_{ST} values compared to other sites ranging from 0.120 at the lowest, to 0.253 (Ch. 3 table 3). Conversely, sites N015 and S012 both resolved as genetically distinct from other sites and had morphological traits that can only be described as extremely average, with no indication of deep genetic divergence from the mean. None the less, their high levels of genetic differentiation from other sites and each other indicates something is inhibiting gene-flow between these sites and others examined.

Population structure between two generations of *F. ovina* from Emorsgate's current stock and its source population shows a divide between the cultivated stock and the wild progenitors, but no specific loci with statistically significant changes in allele frequency related to population identity. Prior work investigating the effects of intentional selection in cultivated lines of *F. ovina* found little reduction in allelic diversity in cultivated stock (Weibull *et al.*, 1991). In the context of *F. ovina*'s established high within-population diversity, resistance to inbreeding even in fragmentary populations (Berge *et al.*, 1998) and the small fraction of loci under selection for slope aspect in the Peaks (twelve SNPs out of over fifteen-thousand), this finding is not entirely unexpected. The findings of Bengtsson *et al.* regarding genetic variation at spatial scales indicated that significant variation occurs at the very local scale due to unequal representation of paternal lineages in the pollen cloud (Bengtsson *et al.*, 2004), however the collection of multiple seed-heads at a distance of over ten metres apart may be sufficient to ensure a varied founding population.

6.2 Genetic underpinnings of climatic adaptation in *F. ovina*

Genetic changes associated with climatic adaptation in plants encompass a great many traits related to the physical management of environmental stresses (Hu *et al.*, 2012; Piveta *et al.*, 2020; Takahashi *et al.*, 2020; T. Zhang *et al.*, 2014), phenology (Bemmels & Anderson, 2019; Matesanz *et al.*, 2010; McKown *et al.*, 2014), reproductive biology (Kottler & Gedan, 2022; Trinder *et al.*, 2020) and the influence of climatic factors on other biotic and abiotic selection pressures (Sardans & Peñuelas, 2004; Sayer *et al.*, 2017; H. Zhang *et al.*, 2020).

The results of chapter 2 of this thesis identify a number of morphological differences between plants originating from north and south facing slope aspect microclimates,

including height, leaf length, above-ground biomass accumulation, tissue density and reproductive investment. The appearance/retention of these traits in common gardens indicates that they are genetically fixed difference rather than plastic responses, and likely evolutionary. The identity of loci associated with selection for slope aspect, increased winter temperatures and drought conditions at the BCCIL and the wider Peak District sites, in chapters 3 and 4, in some cases have direct functional links to these traits. Furthermore, the combination of these morphological traits and genetic loci allows us to infer details about the nature of climatic selection for slope aspect microclimate.

Much of the identified differentiation points to drought stress as a major driving force behind evolution for slope aspect microclimate. Low tissue density is associated with adaptation to drought in a number of plant species (Ryser & Aeschlimann, 1999; Wolfe, 2017), and is a feature of *F. ovina* plants from south facing slopes. Genes differentiated between slope aspects had roles in phosphate transport, a nutrient more limited in droughted soils (Grime & Curtis, 1976; Sardans & Peñuelas, 2004; H. Zhang *et al.*, 2020). Above-ground biomass was significantly higher in plants from north facing slopes, which may be related to differential apportioning of resources to roots vs. shoots, as water stressed plants generally put more resources into root growth (Arsova *et al.*, 2020; Takahashi *et al.*, 2020) and higher root:shoot ratio is strongly correlated with drought tolerance and adaptation (Karcher *et al.*, 2008; Meng *et al.*, 2022; Poorter *et al.*, 2012; Wang *et al.*, 2018). Differentiation was also seen in a gene coding for a rhamnose synthesis gene; rhamnose is involved in both root-sheath mucilage production associated with water stress adaptation (Galloway, Akhtar, *et al.*, 2020; Galloway, Knox, *et al.*, 2020; Liu *et al.*, 2019; Usadel *et al.*, 2004) and as a precursor molecule to pectin in cell wall formation, another possible link to tissue density. An area to target further study into the adaptation of *F. ovina* to slope aspect or drought tolerance might be in root mass or morphology of plants from different slope aspects.

Differentiation was also noted in the allocation of resources to flowering in plants from south facing slopes. Site-by-site variation was high for this trait, but across all sites, plants from south facing slopes had greater numbers of flowering tillers, greater proportions of tillers initiating flowering and greater probability of flowering. No data was collected on timing of anthesis between slope-aspect populations, however work using material from the

BCCIL drought plots noted some variation in flowering time for plants collected from drought treatments, in one of two years (Trinder *et al.*, 2020). This may have some link to the identification of genes in the slope aspect and BCCIL treatment populations of plants, which had significant genetic variation associated with several genes with known links to the timing of flowering initiation and day length (Fornara *et al.*, 2009; Ma & Li, 2018; Sun *et al.*, 2015). Whether these evolutionary changes are in any way related to the discovery of four loci with roles in pollen production showing evidence of selection though is unclear.

Overall, the overlap between functions of genes seen between BCCIL and Peak District sites (see section 4.5) indicates shared responses to manipulated climate and natural microclimatically driven selection. The role of drought stress in driving evolutionary differentiation between north and south facing slopes is well supported, as *F. ovina* commonly inhabits environments where later summer water stress limits growth, is known for its drought tolerance and one locus detected as under selection putatively codes for a calcium signalling gene involved in drought-stress response. However, the comparing results from the Peak District samples with those from the BCCIL suggests winter soil temperature may have an unexpectedly large role in driving slope aspect microclimate response. In support of this, soil temperature is known to be higher throughout the year on south facing slopes (Rorison *et al.*, 1986) and winter is part of the active growing period for *F. ovina* (Grime & Curtis, 1976).

6.3 The conservation of climatically adaptive genetic variation

The work presented in this thesis provides several lines of evidence supporting the importance of microclimate heterogeneity in protecting and conserving climatically adaptive genetic diversity. Here I will discuss their implications for conservation of calcareous grasslands, a biodiverse habitat type recognised as a Biodiversity Action Plan priority habitat (JNCC, 2011). The past 100 years has seen a drastic loss of Britain's species-rich, semi-natural calcareous grasslands, a reduction of 83% land area between 1930 and 2000 (Hooftman & Bullock, 2012). Changes in land use over the last century, particularly the use of nitrogenous fertilisers to "improve" pasture, have degraded most of our low nutrient calcareous grasslands. Remaining CG2 grasslands (Rodwell, 1992) are now largely protected as Sites of Special Scientific Interest (SSSIs), and primarily restricted to steeply sloped hill

and valley sides. Now under threat from climate change, preservation of these habitats must now be a priority for biodiversity in the UK. Some calcareous grasslands, such as that found at the BCCIL, have shown remarkable resistance to the effects of altered climate, while others appear more vulnerable to its effects (Grime *et al.*, 2000, 2008). Factors that predict resistance to climate change appear to include direct effects of habitat heterogeneity (Fridley *et al.*, 2011) and low nutrient status (Grime *et al.*, 2008). Furthermore, within-population genetic diversity has been posited as an important factor in the ability for plant species to resist the effects of climate change, by providing "genetic options" for adaptation with climate (Jump *et al.*, 2009).

The role of microclimatic heterogeneity as a protective factor against climate change is threefold. By providing varying niches, they can foster increased biodiversity which has protective effects at the community and species level (Oldfather *et al.*, 2020). Linked with the previous role, microclimate patches may provide refugia, allowing the retention of key species, preventing local extinction and allowing retention of their contribution to community interactions (Opedal *et al.*, 2015). And thirdly, the presence of microclimatic variation preserves and drives genetic diversity at climatically adaptive loci (Denney *et al.*, 2020; Giaccone *et al.*, 2019; Jump *et al.*, 2008, 2009; Lampei *et al.*, 2019; Ravenscroft *et al.*, 2014). Microclimate heterogeneity capable of driving selection and retaining species can be derived from several sources. Edaphic factors such as soil depth alter water availability and severity of drought (Grime & Curtis, 1976; Prentice *et al.*, 2000, 2015; Ravenscroft *et al.*, 2014). Exposure, altitude and topography alter gene flow, moisture availability and temperature (Günther *et al.*, 2016; Jackson, 1966; Lampei *et al.*, 2019; Mejías *et al.*, 2002; C. Zhang *et al.*, 2017). And, of particular relevance to this study, slope aspect can be considered a major source of microclimatic variation within a habitat (Austin & Van Niel, 2011; Bennie *et al.*, 2006; Chai *et al.*, 2018; Chen *et al.*, 2016; Kimball *et al.*, 2017).

Preservation of plant populations and communities under conditions of altered climate requires microclimate aware habitat management if we are to conserve their adaptive potential. It can be argued that the major aims of conservation interventions is the creation and maintenance of communities and populations that are able to manage themselves, in a self-sustaining manner (Broadhurst *et al.*, 2008; Godefroid *et al.*, 2011; Thomas *et al.*, 2014), as the most economic methods are those with the least input of time and resources. The

data from chapters 2, 3 and 4 show microclimatic variation does drive differential selection for traits and genotypes that provide advantages under varying climatic conditions, in *F. ovina*. Slope aspect microclimate drives changes in morphology that are maintained under altered environmental conditions (common garden), implying a level of genetic fixation for these traits rather than purely plastic responses. The differentiation of these traits in growth habit, tissue density and particularly in reproductive strategy, indicate their importance to success under differing climatic conditions. That the genotypic data from chapter 3 indicates that the majority of these sites are have low levels of genotypic differentiation at neutral polymorphic loci (median F_{ST} = 0.004), vs loci under selection for slope aspect microclimate (median F_{ST} = 0.156) provides further support for the role of these traits in shaping climate-mediated fitness. Similar patterns of F_{ST} associated with loci under selection for microclimate at the BCCIL are seen (median F_{ST} neutral = 0; F_{ST} selected control-drought = 0.368; control-heated = 0.433; drought-heated = 0.356). The BCCIL site occupies a west-facing slope but has a great deal of internal heterogeneity, soil depths varying between under 1cm to greater than 21cm, that have been shown to alter response to, and presumably magnitude of, drought stress (Ravenscroft *et al.*, 2014). The 25 years of climate manipulation has clearly driven adaptive evolution of populations at the treatment level, in agreement with previous work at the site (Ravenscroft *et al.*, 2014, 2015; Trinder *et al.*, 2020). Presumably the initial source of this variation existed in naturally occurring microclimatic and edaphic variation at the site, as well as its situation in a larger, heterogeneous landscape (the Peak District). The magnitude of changes seen in such a short time-period may be argued to provide at least anecdotal support for the hypothesis that standing, microclimate-driven genetic variation, can indeed spread through populations in the event of climatic change, driving hastened evolutionary responses and protecting them from deleterious effects.

These data on the presence, distribution, importance, speed and ability to spread of climatically adaptive genetic variation form microclimate sites however raise concerns about the potential effect propagation of seed for conservation may have on population genetics. Propagation of grasses for seed, notably in the form of grain crops, has altered seeding traits as there's an extremely strong selection pressure for traits that improve harvesting of seed for replanting (Hillman & Davies, 1990). Cultural conditions of *Clarkia*

pulchella for conservation propagation have been shown to negatively affect population level adaptation to adverse climate and general hardiness compared with progenitor stock after only eight generation (Pizza *et al.*, 2021). However, due to the mating system and wide range of environmental tolerances displayed by *F. ovina*, active selection for particular traits in commercial strains has been shown to have limited effects on genotypic diversity (Weibull *et al.*, 1991), and under open-pollination in mesic conditions, *F. ovina* may be expected to show relatively low rates of allele loss. This is what was found in analysis of the population of *F. ovina* grown by Emorsgate Seeds – though all three populations (fourth generation stock, sixth generation stock and Grass Wood source population) showed high levels of variation from each other, median F_{ST} for all SNPs remained at 0.020, and no loci could be significantly in associated with selection for conditions at the Emorsgate Seeds site in Norfolk. Though some, non-significant, changes in allelic frequency were seen, no single SNP was biallelic in the Grass Wood population but monoallelic in the fourth and sixth generations, indicating retention of allelic variants after six generations of cultivation.

Synthesising these results, I come to the following recommendations regarding future practice in conservation of calcareous grassland: (i) that assessment of the potential threat to individual grasslands under climate change includes integrating range and structure of microclimatic heterogeneity as an important source of extant adaptive potential; (ii) that in designating the boundaries and locations of protected areas, consideration is given to including and protecting topographic features likely to represent microclimate islands of climatically adaptive genetic variation; and (iii) reseeding operations consider combining the use of commercially propagated *F. ovina* seed with small numbers of individuals collected from and planted at diverse, available microclimate sites. The aim of which acknowledges the fact that genetic diversity is well retained in commercially propagated populations, these may be collected from a single site that has been under selection for particular climatic conditions. In order to reliably generate robust, genetically varied populations with increased adaptive potential, addition of plants from varied microclimate sites, and planting across available microclimatic sites, may have benefit in allowing populations to adapt more rapidly to altered climatic conditions.

6.4 Bibliography

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