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Development and maintenance of genetic diversity in Scots pine, *Pinus sylvestris* (L.)

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Thesis abstract

Forests are among the most important repositories of terrestrial biodiversity and provide a broad range of ecosystem services. During millennia, forests have changed, adapted and evolved under changing conditions. However, in the present century, forests are facing environmental changes at rates with no precedents. A major concern is the risk of declining forest genetic diversity, since genetic variation as the raw material underpinning adaptation is key in maintaining the resilience of forest ecosystems against environmental changes. Understanding the different processes responsible for developing and maintaining the genetic diversity of tree species is essential to better predict tree responses under new conditions. Therefore, this thesis aimed to determine how different forces interact to shape and maintain within and among population genetic diversity of Scots pine and what the implications are for conservation and management under forthcoming environmental conditions. From local to continental scales, we followed a multilevel approach, and found that (i) historic climate changes and geographical barriers have played an important role in shaping the extent and spatial distribution of current genetic diversity of Scots pine. Despite contemporary habitat reduction and fragmentation we found that (ii) high levels of neutral genetic diversity remain in the Scottish populations of Scots pine, with gene flow and specifically wind-driven gene flow dominating over genetic drift and preventing differentiation among the Scottish populations. However, (iii) considerable impacts in the spatial distribution of genetic variation have occurred as a consequence of intensive historical forest management practices. Furthermore, we found that (iv) substantial levels of adaptive genetic variation are present in the Scottish populations of Scots pine, likely a result of selective processes resulting from the different environments they live in, with highly heritable traits, although similar capacity for response through phenotypic plasticity to warming. Our results help to further disentangle the forces maintaining

genetic diversity in one of most widespread conifers in the world, and improving predictions of likely range shifts and adaptation of the species in response to contemporary changes. We provide some recommendations to conservation and management practices.

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and compared by AICc to assess the importance of Population. The best models were determined by AICc, and the presence (+) or absence (-) of fixed effects are indicated for each of the traits listed. ΔAICc values represent the difference in AICc between the null model and the best (a value of zero indicates that the null model was the most parsimonious)

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Chapter 1

General introduction

1.1 Forest genetic diversity in a changing environment

Forests are among the most important repositories of terrestrial biodiversity, and currently they cover the 30 percent of the global land area (FAO 2010). They provide a broad range of ecosystem services, including providing habitat for many species, watershed protection, erosion prevention and acting as carbon sinks, as well as providing a wide range of livelihoods for people (Milennium Ecosystem Assessment, 2005). The enormous range of goods and services provided by trees and forests is both a function of and testimony to the genetic diversity contained within them (FAO 2014a).

Forest genetic diversity can be expressed as any variation in all aspects of the tree structure and represents the genetic variation among trees (Ennos et al. 1998). Forest genetic diversity plays an essential role in underpinning forest resilience by facilitating evolutionary processes, and it is key in forest responses to environmental changes and disturbances, such as habitat loss, fragmentation or pathogen attack (DeSalle & Amato 2004, Schaberg et al. 2008, Alfaro et al. 2014, Cavers & Cottrell 2014, Ellegren & Ellegren 2016, Fady, Aravanopoulos, et al. 2016). In particular, the existence of a high level of genetic diversity within stands is a prerequisite for forest trees to adapt and be resilient to the unpredictable effects of a changing environment. Conversely, reduced levels of variability are seen as limiting a species' ability to respond to these changes in both the long and short term (Jump & Penuelas 2005).

Over time forests have changed, adapted and evolved under changing conditions, and those changes have shaped the levels of genetic variation found within them. For instance, historical migrations as a consequence of past climate changes had a strong effect in shaping spatial patterns of genetic diversity (Petit et al. 2003). Trees are generally long-lived and have developed natural mechanisms to maintain high levels of genetic variation (i.e. high rates of outcrossing or long-distance gene flow and the resultant reproduction among unrelated distant individuals). These mechanisms, combined with the fact that trees, as well as most other organisms, live in contrasting environments, and usually undergo differential selective pressures, will set up the context for adaptation to occur and determine the levels and extent of genetic variation. Genetic variability will be crucial for adaptation to climatic regimes different from those in which they have evolved (FAO 2014a).

In the present century, European forests are facing numerous threats, including habitat destruction, fragmentation, pollution, climate change, poor silvicultural practices or the use of low quality of poorly adapted forest reproductive material (Koskela & Buck 2007). Furthermore, those environmental changes are occurring at an unprecedented rate and magnitude (IPCC 2013) which is predicted to intensify in the future with major effects on biodiversity (Alfaro et al. 2014). It has been estimated that over 15 billion trees are cut down each year (Crowther et al. 2015), which means that approximately 13 million hectares of natural forest are lost every year worldwide (FAO, 2010). However,

the rate of net forest loss has been cut by over 50 percent during the last 25 years, due to sustainable forest management, trees establish due to land abandonment. All those environmental changes might challenge the adaptability of long-living forest tree species. Some studies have already shown the impact that intensive forest management practices can have on forest genetic diversity, such us changes in gene frequencies (Schaberg et al. 2008), loss of alleles (Adams et al., 1998; Rajora et al., 2000; Kettle et al., 2007; Ortego et al., 2010), or changes in the spatial organisation of genetic variation (Piotti et al. 2013, Sjölund & Jump 2015). Global change is also altering growth and mortality patterns (e.g. Galiano et al. 2010, Vilà-Cabrera et al. 2012), thereby likely altering species distribution ranges over the coming decades (e.g. Thuiller et al. 2005). Species distribution ranges might be altered even when genetic diversity is considered (Benito Garzón et al. 2011, Oney et al. 2013). Furthermore, global change might have unpredictable changes in the adaptive capacity of species which might result in decreasing their ability to resist and recover from further environmental perturbations (Jump & Penuelas 2005). Consequently, reductions in diversity due to environmental changes or human activities might impact species resilience and increase the threat from pest and diseases or maladaptation (Cavers & Cottrell 2014).

The importance of including genetic diversity features in sustainable forest management has therefore been widely argued in recent years (Lefèvre et al. 2013, FAO 2014a, Alfaro et al. 2014, Cavers & Cottrell 2014, Fady, Cottrell, et al. 2016) particularly

for marginal populations (Fady, Aravanopoulos, et al. 2016). Accordingly, several schemes have been proposed for monitoring genetic diversity at the European (Aravanopoulos et al. 2015) and international levels (Graudal et al. 2014), including the quantification of both adaptive and neutral genetic diversity in forest trees. Maintaining high levels of genetic variation will be essential for global change mitigation and adaptation. As a consequence, forest practices that maintain genetic diversity over the long term will be required as an integral component of sustainable forest management (FAO 2014a).

Given almost universal recognition of the importance of including genetic variability in forest management, it is crucial to understand which processes shape and maintain forest genetic diversity and how the major forces of these processes interact. Therefore, understanding the different processes responsible for the development and maintainance of the genetic diversity of tree populations is essential to better predict tree responses under new conditions.

1.2 Processes influencing forest genetic diversity

To monitor forest genetic resources and support the development of conservation strategies it is important to understand the amount and distribution of neutral and adaptive genetic diversity. Neutral and adaptive genetic diversity do not necessary show the same diversity pattern, nor provide the same information, as they are shaped

by different processes. **Neutral variation** has little or no effect on the phenotype of the tree or on its performance (Ennos et al. 1998). It is characterized by a slower rate of evolution of neutral sites and can reveal information about, for example, the breeding system of the species, gene flow among and within populations, and the history of populations. However, neutral diversity cannot inform about natural selection (Mason, 2004). **Adaptive genetic variation** underlies differences in the phenotype and thus, performance and fitness of trees (Ennos et al. 1998). It is selectively important, and it manifests variation in ecologically important characters such as phenology, growth, morphology or pest resistance (Ennos et al. 1998). In contrast with neutral evolution, adaptive variation can appear within one or a few generations (Petit et al. 2008), and traditionally is detected in provenance and progeny trials and reciprocal transfer experiments, where environmental variation can be controlled, thereby exposing the genetic contribution to phenotypic variation.

The levels and distribution of genetic variation results from the joined action of migration, mutation, selection and drift (Loveless & Hamrick 1984). Thus, neutral variation may be governed mostly by mutation, migration and drift, whereas variation at quantitative traits, or adaptive variation, should depend on a balance between mutation, migration and selection (Karhu 2001). However, other factors might also shape genetic variation, such us the breeding system, dispersal mode (i.e. wind or

animal dispersed, etc), mode of reproduction (i.e. sexual, asexual) or the effective population size.

Fluctuations in the numbers of alleles in a population between generations, are referred to as **genetic drift**. Typically, genetic drift occurs in small populations, where infrequently-occurring alleles face a greater chance of being lost. The ultimate consequence of continuous genetic drift is that alleles are either lost from a population or driven to fixation. Both possibilities decrease the genetic diversity of a population. **Migration** is the movement of genes or organisms from one location to another. Since tree individuals are non-motile, migration happens through seed and pollen flow. If the migrating genes mate with the destination individuals, they can alter the existing proportion of alleles in the destination population. **Mutation** refers to the permanent alteration of the nucleotide sequence of the genome as a consequence of errors during DNA duplication or other types of DNA damage (e.g. due to environmental factors such us ultraviolet radiation or chemicals). **Selection** indicates the differential survival and reproduction of individuals due to differences in the phenotype.

There is a more developed understanding of the links between such evolutionary processes and the levels and maintenance of genetic variation than the external forces governing those processes. However, as rapid anthropogenic environmental change is altering selection pressures on natural plant populations, some additional external

pressures, such us environmental variation (i.e. variation in geography or presence of fragmentation), changes in climate or changes in management, might influence such evolutionary processes and therefore the levels and maintenance of genetic variation at different temporal and spatial scales (Figure 1.1).

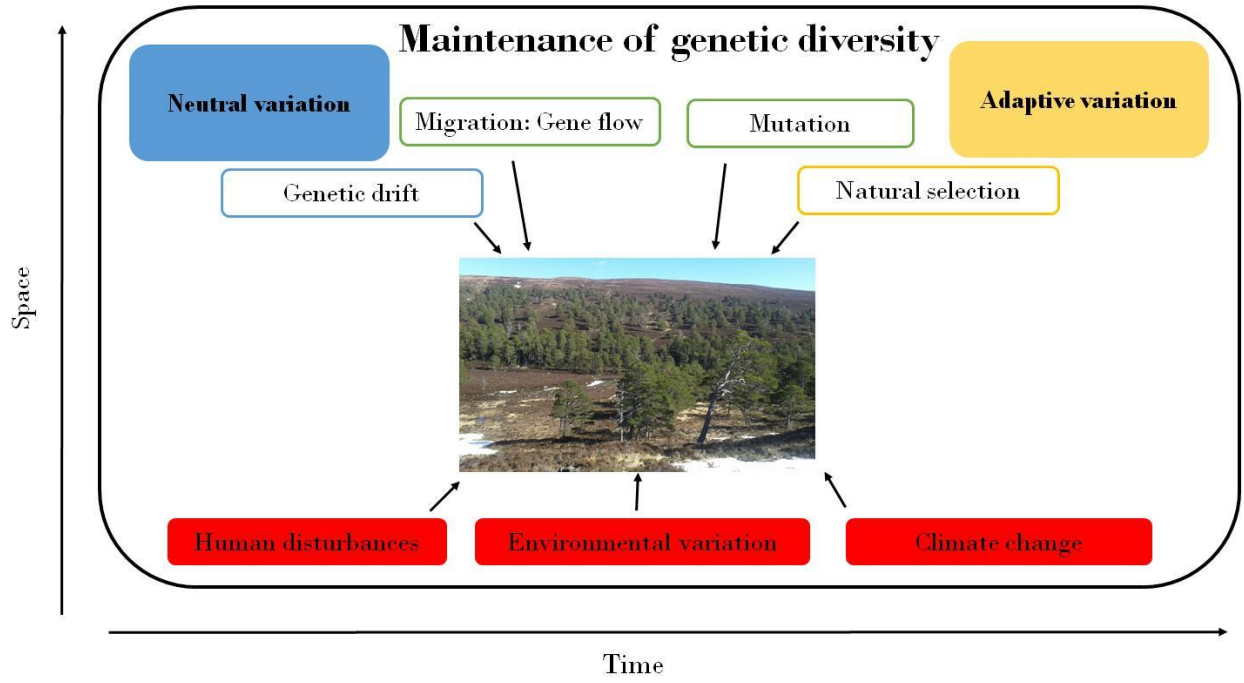

Figure 1.1 Processes influencing neutral and adaptive genetic variation.

Environmental variation- Variation in geography

The genetic diversity and genetic structure of tree populations, and the forces shaping gene flow within and between populations, are influenced by the landscapes they occur within. Geographic heterogeneity exists everywhere within nature and the landscapes they occur in, and are distributed neither uniformly nor randomly but structured in space and time (Loveless & Hamrick 1984, Legendre 1993). Geographic heterogeneity

occurs over a range of spatial and temporal scales and may imply different selection pressures resulting in diverse adaptations. Geographic heterogeneity can also imply physical barriers, such us mountains (Naydenov et al. 2011) or oceans, or fragmentation (Provan et al. 2007, Cuartas-Hernández et al. 2010) that may even restrict gene flow. However, in some cases, increases of pollen flow counteract diversity loss resulting from fragmentation (White et al. 2002, Petit & Hampe 2006, Wang et al. 2012, Davies et al. 2013). Geographic heterogeneity may be also accompanied by different levels of competition, predation, or pathogen prevalence.

Environmental variation- Variation in climate

Climate changes over time. Climatic fluctuations over the Earth´s surface have modelled actual distribution of trees, and therefore patterns of genetic composition of trees. Specifically, climatic conditions in the Late Quaternary, which peaked with the major cold event 26,000-19,000 years ago (Chiverrell & Thomas 2010), caused a large part of the Northern Hemisphere to be covered by ice or become otherwise uninhabitable (Willis 2000a). Temperatures in some areas of Northern Europe were 10-20°C cooler than the present (Barron & Pollard 2002, Annan & Hargreaves 2013) (Figure 1.2). These conditions restricted most tree species to patchy, discontinuous and climatically constrained areas designated as **glacial refugia** (Cheddadi et al. 2006). Such conditions in climate and the following migrations once conditions became favorable

have influenced subsequent levels and distribution of genetic variation (Hewitt 1996, 2000)

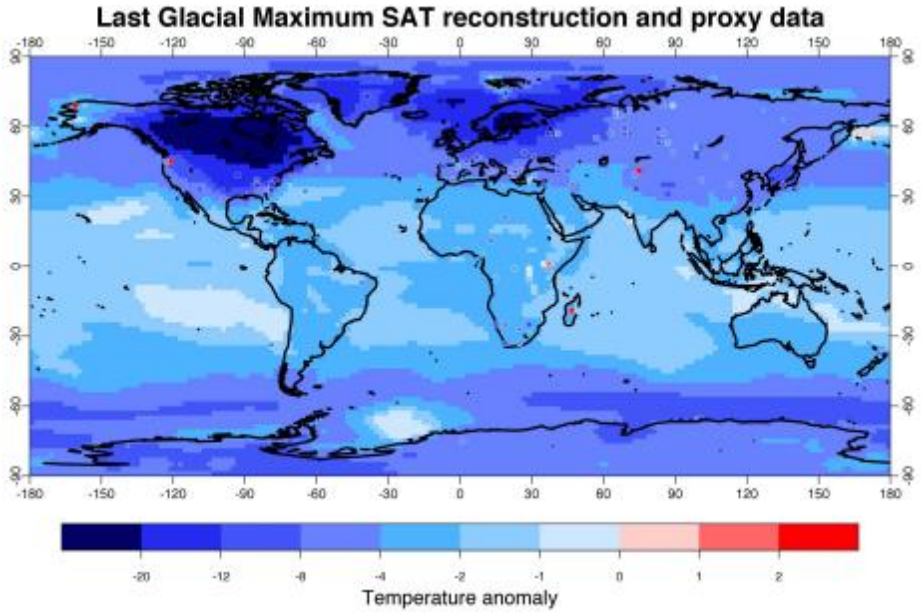

Figure 1.2 Temperature anomaly at Last Glacial Maximum (LGM) (taken from (Annan & Hargreaves 2013))

Climatic changes and species range shifts have been a recurrent phenomenon throughout the history of Earth (Hampe & Jump 2011). Recent changes in climate may also impose different selection pressures on plant species resulting in diverse adaptations to new conditions. During the last century, global mean temperature has increased substantially (Figure 1.3) and, if greenhouse gas emissions continue at current rates, it is predicted to continue to rise through the 21st Century (IPCC, 2013). Furthermore, these changes will be accompanied by an alteration of precipitation patterns and an increase on the frequency of extreme events (i.e. torrential rainfall or

severe drought) (IPCC, 2013). Changes in global climate are therefore likely to have important consequences for species persistence and for forest population dynamics at different scales (Peñuelas & Boada 2003, van Mantgem et al. 2009, Matías & Jump 2015, Matías et al. 2016). Forecasting the impact of a particular change in climate on individual tree species has been complex, as the local outcome of global changes is hard to predict. Furthermore, the rate of climate changes or the presence of extreme events, might lead to rapid and directional changes in allele frequency within populations, whose outcome is difficult to anticipate (Jump et al. 2009).

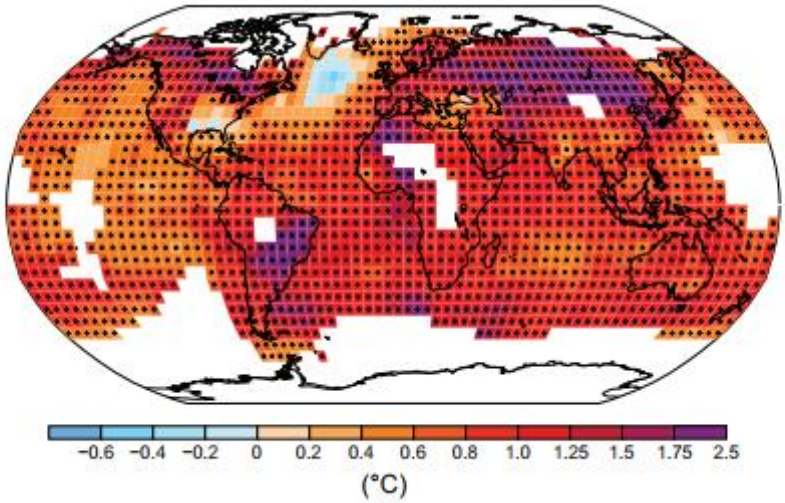

Figure 1.3 Observed change in surface temperature from 1901 until 2012 (IPCC, 2013).

Climate also changes over space. The existence of genetic variability associated with climatic variation, such as temperature, light, wind or water availability, at the intraspecific level has been detected for many species both among and within populations (e.g. (Martínez-Vilalta et al. 2009, Matías et al. 2014)). Furthermore, climate

might also influence genetic variation by limiting migration, or even promoting contrasting flowering timing in some areas and leading to mating asynchronies (Whittet et al. 2017).

Human influence

Anthropogenic impacts remain the leading cause of deforestation, with changes in land use being the primary driver. Deforestation, fragmentation and forest exploitation through forest practices such us logging or thinning have promoted a widespread modification of Europe's forests, impacting genetic diversity within and among populations (FAO 2014a). It is thought that intensive forest exploitation influences the evolutionary processes of selection, drift, gene flow and mutation, sometimes increasing diversity, as in the case of domestication, but often reducing it (Ledig 1992), promoting changes in gene frequencies (Schaberg et al. 2008) or losses of alleles (Adams et al., 1998; Rajora et al., 2000; Kettle et al., 2007; Ortego et al., 2010). However, we lack a clear understanding of the genetic impacts of forest exploitation and fragmentation (Young et al. 1996, Rajora & Pluhar 2003, Bradshaw 2004, Schaberg et al. 2008, García-Gil et al. 2015). Therefore, a better knowledge of the effects of forest practices on forest genetic diversity is needed.

1.3 The study case: Scots pine

Pinus sylvestris L. is a wind-pollinated outcrossing conifer with the most widespread distribution of the genus. Its range covers Eurasia from the northern Fennoscandia in the North in Norway to the South of Spain and South of Turkey and from the West of Scotland, to Asia in the East (Carlisle & Brown 1968) (Figure 1.4). It is a very valuable species, both economically and ecologically. Its strong timber is frequently used in construction and industry. Scots pine forest provides a range of habitat for numerous species, as the case of the endangered capercaillie (*Tetrao urogallus*) or twinflower (*Linnea borealis*). Scots pine is a monoecious species, with male and female flowers found on the same tree. Male inflorescences appear in large groups, and their colour varies from yellow to violet-yellow, usually in the lower branches, whereas female flowers are small, red-purple and globular, usually in the upper branches. The reproductive system of pines is exclusively sexual and both pollen and seeds possess adaptations for wind dispersal.

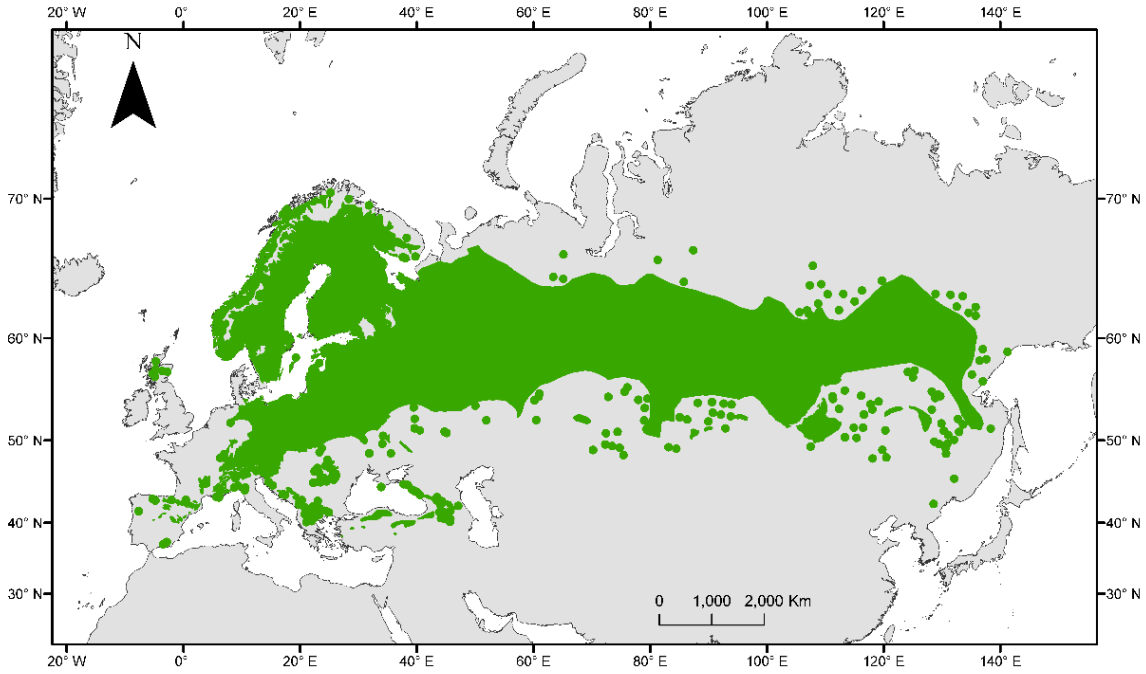

Figure 1.4 Scots pine global distribution. Presence data obtained from EUFORGEN database. Green areas represent natural presence of Scots pine.

At global scales, a considerable amount of prior research has focussed on the levels of neutral genetic variation in this species (Table 1.1). Most of these studies involve two approaches, either separately or in combination: (a) descriptive analyses of the levels and/or structure of genetic variation and/or (b) phylogeographical analysis.

Most studies of diversity have shown that, in general, Scots pine populations are not genetically impoverished (Provan et al. 1998, Robledo-Arnuncio et al. 2005, Wachowiak et al. 2011, 2013, Pavia et al. 2014, García-Gil et al. 2015) and that the partitioning of neutral genetic variation is most often found within populations, with a much smaller component due to differences between them (Provan et al. 1998, Robledo-Arnuncio et

al. 2005, González-Díaz et al. 2017, Tóth et al. 2017). Several studies addressing phylogeographical analysis have proposed that Scots pine retreated to the southern European peninsulas during the Last Glacial Maximum (LGM) (Prus-Glowacki & Stephan 1994, Sinclair et al. 1999, Soranzo et al. 2000, Cheddadi et al. 2006, Labra et al. 2006, Naydenov et al. 2007, Bilgen & Kaya 2007, Scalfi et al. 2009, Belletti et al. 2012, Prus-Glowacki et al. 2012, Dering et al. 2017). However, an increasing body of evidence suggests that not only southern but also sparse and mid-northern refugia might have contributed to the Holocene expansion of Scots pine (Willis et al. 2000, Willis & van Andel 2004, Cheddadi et al. 2006, Naydenov et al. 2007, Pyhäjärvi et al. 2008, Prus-Glowacki et al. 2012, Parducci et al. 2012, Buchovska et al. 2013, Bernhardsson et al. 2016, Dering et al. 2017, Tóth et al. 2017). Consequently, several migration routes have been proposed which might have crossed the continent in several fronts, a western (Pyhäjärvi et al. 2008, Dering et al. 2017, Tóth et al. 2017) and an eastern font (Naydenov et al. 2007, Buchovska et al. 2013, Tzedakis et al. 2013).

Table 1.1 Overview of neutral genetic diversity studies with Scots pine

Genome region	Molecular marker	Location	Nm	Np	Ni	Diversity	H	FST or GST	Reference
		Sweeden	11	5	-	0.307	-	-	Rudin et al., 1974
		Poland	12	-	22	0.321	-	-	Krzakowa et al. 1997
		Poland, German, Hungary, Turkey	3	19	-	0.313	-	-	Mejnartowicz 1979
		Sweeden	9	3	-	0.303	-	-	Gullberg et al. 1982
		Sweeden	11	9	-	0.31	-	-	Gullberg et al. 1985
		Scotland	16	14	-	0.309	-	-	Kinloch et al. 1986
		Sweeden	13	3	-	0.283	-	-	Muona & Harju 1989
	Allozyme	Sweeden, China	14	7	-	0.211	-	-	Wang et al. 1991
		Sweeden, Turkey	14	16	-	0.235	-	-	Szmidt & Wang 1993
		Switzerland	11	3	-	0.271	-	-	Neet-Sarqueda 1994
		Latvia, Ukraine, Russia	21	18	-	0.273	-	-	Goncharenko et al. 1994
		Eastern Europe, Turkey	8	13	-	0.356	-	-	Prus-Glowacki & Bernard 1994
		Poland	7	5	-	0.344	-	-	Szweykowski et al 1994
		Poland	-	-	-	-	-	-	Chybicki et al. 2008
		North Europe	14	2	494	0.371-0.374	-	-	Prus-Glowacki et al. 1993
		Spain, E and N Europe		8+16	-	-	-	0.040	Prus-Glowacki & Stpehan 1994
	Isoenzymes	Spain, France	-	14&2	-	0.302-0.311	-	0.042	Prus-Glowacki et al. 2003
		Europe	10	51	-	-	-	-	Prus-Glowacki et al. 2012
		Turkey	10	6	-	-	-	-	Bilgen & Kaya 2007
		Scotland		41	6705	-	-	-	Forrest et al. 1980
		Norway, Sweeden, Poland, Cz, Scotland		6	-	-	-	-	Forrest et al. 1982
	Monoterpene	Scotland		-	-	-	-	-	Kinloch et al. 1986
		Turkey		9	-	-	-	-	Semiz et al. 2007
		Bulgaria		12	432	-	11	0.088	Naydenov et al. 2005
		Greece, Italy	1	7	-	0.411	-	0.222	Powel et al. 1995
		Scotland	13	15	330	0.969	174	-	Provan et al. 1998
		Bulgaria	6	12	432	40.93	134	0.049/0.064	Naydenov et al. 2005
		Spain	6	13	322	0.978	139	0.024	Robledo-Arnuncio et al. 2005
		Europe	7	35	1380	-	178	0.060	Chedaddi et al. 2006
cpDNA	SSR	Italy	2	4	96	0.92	17	0.140	Scalfi et al. 2009
		Spain	6	30	706	-	307	0.070/0.191	Soto et al., 2010
		Portugal, Spain, Central Europe	5	6	96	-	56	-	Pavia et al. 2014
		Sweeden	11	3	571	0.56	-	-	Garcia-Gil et al. 2015
		Romania	14	13	326	0.44	-	0.120	Bernhardsson et al. 2016
		Scotland	5	18	469	0.931	64	-	Gonzalez-Diaz et al. under review
		Carpathian rerion	4	20	421	0.546	141	0.074	Toth et al. 2017
		Europe	13	92	1384	0.786	73	0.015	Dering et al., 2017

mtDNA	RFLP	Scotland	-	20	466	0.12	3	0.37	Sinclair et al., 1998
		Europe	-	38	762		4	-	Sinclair et al., 1999
	Sequence polimorphism	Scotland	1 region	23	747		2	0.59	Soranzo et al. 2000
		Italy	1 region	10	87	-	2	-	Labra et al. 2006
		Europe	1 region	106	1380	-	3	0.80	Chedaddi et al. 2006
		Eurasia	2 region	54	986	0.141	3	0.657	Naydenov et al. 2007
		Europe	2 region	37	714	0.200	4	0.655	Pyhajarvi et al. 2008
		Eastern Europe, Ural mountains	2 region	54	474	0.300	2	0.239	Bukovska 2013
		Sweeden	1 region	3	554	0.240	-	-	Garcia-Gil, 2015
		Romania	2 region	13	326	0.200	3	0.196	Bernhardsson, 2016
		Europe	2 region	92	1384	0.131	5	0.628	Dering et al., 2017
	SNP	Scotland	2 region	12	120		1	-	Wachowiak et al., 2013
nDNA	ISSR	Italy	8	10	87	0.264	-	-	Labra et al 2006
	SSR	Italy	3	4	96	0.810	-	0.080	Scalfi et al. 2009
		Bulgaria	6	12	36	0.622	-	0.154	Naydenov et al. 2011
		Italy	9	21	449	0.847	-	0.058	Belleti et al. 2012
		Norway	3	1	57	0.853	-	-	Nowakowska et al., 2014
		Portugal, Spain, Central Europe	4	6	96	0.910		0.100	Pavia et a., 2014
		Sweeden	8	3	581	0.790	-	-	Garcia-Gil et al. 2015
		Romania	10	13	324	0.550	-	0.056	Bernhardsson et al. 2016
		Scotland	6	18	540	0.607	-	0.019	Gonzalez-Diaz et al., under review
		Carpathian region	8	20	421	0.586	-	0.071	Toth et al. 2017
		Scotland and Siberia	3	3	777	0.574/0.579		0.004/0.035	Gonzalez-Diaz et al., 2017
	SNP	Europe	16	8	40	-	-	-0.030 - 0.140	Pyhajarvi et al. 2007
		Scotland	12	21	42	0.0078	-	-	Wachowiak et al., 2011
		Scotland	-	12	120	0.0098	-	0.010	Wachowiak et al., 2013

Nm refers to Number of markers / loci (region with variation/region tested), Np refers to number of populations, Ni refers to number of individuals, Diversity refers to (He or nucleotide diversity). H refers to Number of haplotypes / terpenes.

Furthermore, studies focussing on adaptive variation in Scots pine were developed as early as the beginning of the 20th Century by researchers associated with the International Union of Forest Research Organisations (IUFRO), where provenance trails from Eurasia were performed (Giertych 1979). Differences were found among provenances in height growth, resistance to frost or diseases, anatomical structures or wood quality, among others (Giertych & Oleksyn 1992). For many years, extensive studies have documented variation between populations throughout its distribution. Thus, numerous studies found differences among latitudinal populations in traits related to timing of growth (Notivol et al. 2007, Garcia-Gil et al. 2003, Clapham et al. 2002), cold resistance (Wachowiak et al. 2009, Hurme et al. 1997, Aho 1994), light spectra (Ranade & García-Gil 2013) or drought response (Matías et al. 2014). But also differences in relation to frost hardiness have been found between populations separated on a longitudinal gradient (Andersson & Fedorkov 2004).

In Britain, native Scots pine populations are restricted to Scotland. Here, Scots pine is the iconic species of the Caledonian pinewoods, which are recognized as descendants of the original forests that once occupied the British Isles after the last Ice Age, covering a total area of 18,000 ha. Nowadays, native Scots pine forests in Scotland occupy only 1% of the area that they used to cover in the past, scattered over 84 fragments (Mason, 2004) (Figure 1.5) where they represent the North-West limit of the species distribution. Several millennia of isolation with respect to continental European populations raise the

possibility that these Scottish forests might harbor local adaptations and constitute valuable forest genetic resources. Scots pine is unlikely to face extinction at species level across its distribution as a result of predicted climate change. Nevertheless, small or fragmented populations, such as those in Scotland, might be at risk of decline. Furthermore, several questions remain about the origins of Scots pine populations in Scotland (UK), for which multiple colonisation sources have been suggested (Forrest 1980, Bennett 1984, 1995, Kinloch et al. 1986, Sinclair et al. 1998, Provan et al. 1998).

The differential contribution of processes determining distribution of genetic variation in Scots pine (i.e. migration, mutation, selection and drift) and the external forces shaping such processes (i.e. geographic variation, climatic variation and human interference) at different temporal and spatial scales remain poorly understood. Understanding the different processes responsible for developing and maintaining the genetic diversity of Scots pine will be essential to better predict tree responses under new conditions.

Figure 1.5 Example of some Scots pine populations from Scotland studied in the present thesis. Top left: Beinn Eighe; top right: Shieldaig; middle left: Glenn Affric; middle right: Abernethy; bottom left: Glen Derry; bottom right: Glen Tanar. [Photographs by P. González-Díaz]

1.4 Thesis outline

The general aim of this thesis is to determine how different forces interact to shape and maintain within and among population genetic diversity of Scots pine and what the implications are for conservation and management under forthcoming environmental conditions.

After this first introductory chapter, four research-based chapters are presented (see Table 1.2 for details of research-based chapters), each in manuscript format before a concluding general discussion (Chapter 6). The focus of these chapters is as follows:

Chapter 2: Understanding the role of past climate changes and geographical barriers in the genetic structure of Scots pine

The aim of this chapter is to disentangle how historic **climate** changes and **geographical** barriers have influenced **past demographic processes** and consequent genetic structure of Scots pine at the European scale. We combined genetic and palaeoenviromental data to assess past demographic processes and consequent genetic structure. Firstly, we used an approximate Bayesian computation framework to determine the role of refugial populations to subsequent Holocene migration and main colonisation sources for central and northern European populations. Secondly, we used a species distribution modelling approach to determine the most likely suitable areas for the survival of the species during the historical climate changes (i.e. Last Glacial Maximum).

Chapter 3: Weak isolation by distance and geographic diversity gradients persist in Scottish relict pine forest, potentially linked to flowering asynchrony and effective gene flow

The aim of this chapter is to assess how **gene flow** and **genetic drift** interact to maintain genetic diversity at a regional scale. We examined patterns of genetic diversity within and among populations across Scotland to investigate the levels of diversity and the existence of trend of isolation by distance. We used gene flow networks to investigate any directionality in the patterns.

Chapter 4: Ecology and management history drive spatial genetic structure in Scots pine

The aim of this chapter is to investigate the effects of **historical and contemporary forest management**, characterized by intense felling and natural regeneration respectively, on genetic diversity and fine-scale SGS in adult and juvenile cohorts. We examined patterns of genetic diversity, both levels and structure, of fragmented Scots pine stands in the Scottish Highlands, and compared them with a remote, unmanaged stand. We also compared the forest age structure by using core data.

Chapter 5: Scots pine from a heterogeneous Scottish landscape grown under controlled environment conditions show substantial intra-population adaptive diversity but a consistent response to a simulated warmer climate.

The aim of this chapter is to characterize the **intraspecific adaptive variation** in Scots pine early growth traits (seed mass, seedling emergence, biomass accumulation and key

above & below traits) among and within populations, relate this to spatial variation in home site **environment**, and estimate the likely response of those populations to projected **climate change**. We used a common garden approach under strictly-controlled conditions with two temperature treatments, current temperature and increased temperature. We used linear mixed models to assess the level and extent of traits variation and linear regression to analyse germination ratios.

Table 1.2 General overview of the thesis, specifying objectives, methods and results in form of publications resulted from each of the thesis chapter.

Chapter	Objective	Methods					Result
		Study area	**Data Type**	**Statistical method**	**Temporal scale**	**Spatial scale**	
2	To dissentangle how historic **climate** changes and **geographical** barriers have influenced past demographic processes and consequent genetic structure	Europe & Scotland	nSSR cpSSR SNP	Approximate Bayesian Computation	Past & Present	Continental	González-Díaz et al. (under preparation) Expected to be sent to *Molecular Ecology*
			Ocurrence data (EUFORGEN, TSDE, IFN)	Species distribution models			
3	To assess how **gene flow** and **genetic drift** interact to maintain genetic diversity	Scotland	nSSR cpSSR	Common test, bayesian clustering, gene flow networks	Present	Regional	González-Díaz et al. (under review) *iForest*
4	To understand the effects of historical and contemporary **forest management practices** in the spatial distribution and levels of genetic variation	Scotland & Siberia	nSSR	Common test, spatial autocorrelograms	Past & Present	Local	González-Díaz et al., 2017. *Forest Ecology and Managment* DOI: 10.1016/j.foreco.2017.05.035
			d.b.h. and trunk cores	Linear model			
5	To characterize the intraspecific adaptive variation in Scots pine early growth traits among and within populations, relate this to spatial variation in home site **environment**, and estimate the likely response of those populations to projected **climate** change	Scotland	Germination	Logisitic regresssion	Present & Future	Local & Regional	González-Díaz et al. (under preparation) *Expected to be sent to Tree Physiology*
			Other adaptive traits	Mixed effect models			

Published papers are indicated with relevant DOI numbers

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Chapter 2

Understanding the role of past climate changes and geographical barriers in the genetic structure of Scots pine

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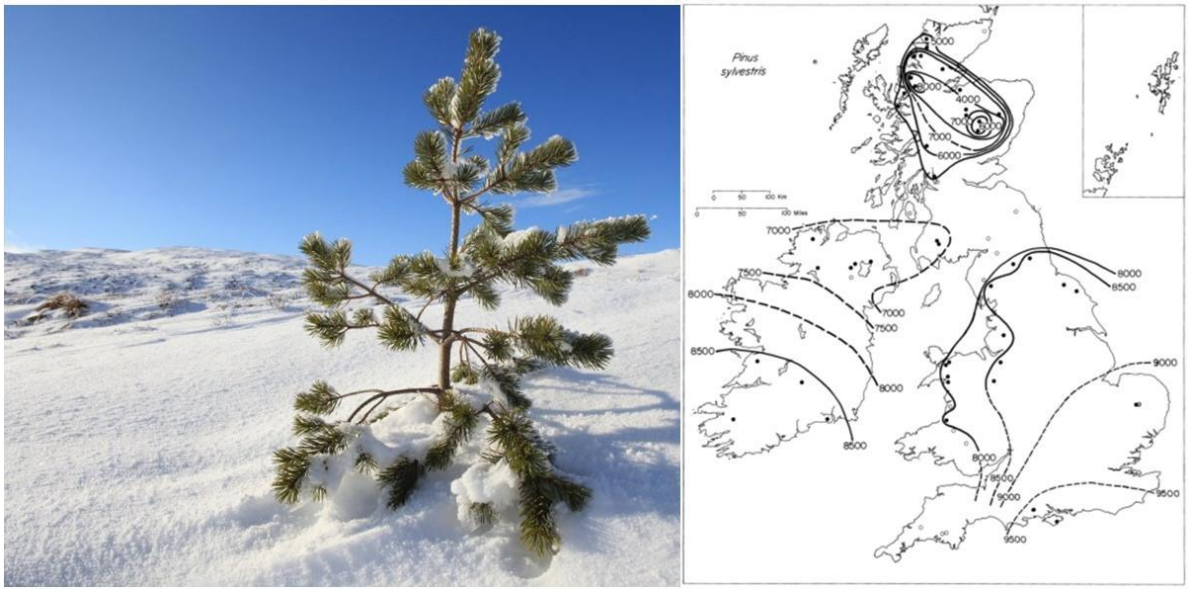

Intention to submit: Molecular Ecology

Photos in Chapter 2 main page taken from, left: https://treesforlife.org.uk/ and right: Birks, 1989.

2.1Abstract

Migrations patterns of tree species in response to past climate changes have been a subject of increased debate during the last decades. However, traditional refugia during glacial maximum and subsequent migrations are likely to have been an oversimplification as environmental heterogeneity and geographic complexity might have created complex species persistence and migration scenarios. Persistence of the species in small northern refugia would have significant implications for subsequent patterns of colonization, and more rigorous testing of hypothesis on the location of glacial refugia, Holocene migration routes and associated demographic events is needed. Here, we investigated migration and colonization routes and the main processes shaping such migrations of European Scots pine, which is one of the most widespread tree species in the world and has high ecological and economic value. We use a complementary use of phylogeographic reconstructions and Species Distribution Models (SDM), which can deliver a robust analysis of past colonization ranges and the putative locations of refugial populations. Our results supported (1) similar and even greater diversity in northern populations of Scots pine in comparison with southern peninsular refugia; (2) a differentiated role of peninsular glacial refugia in contributing to Holocene colonisation: Italian and Asia Minor peninsulas might have not contributed to Holocene migrations whereas Iberian and Balkan peninsulas might have, being the latter the most likely source for central-northern European populations, and (3) North-Western populations likely derived

from two genetic pools and underwent a significant bottleneck event around the time of the LGM. Our findings provide a greater resolution in the role of refuge areas into Holocene migrations of Scots pine and likely sources of colonization and suggest that historic climate changes and geographical barriers have played an important role in the spatial distribution of current genetic diversity of Scots pine. Furthermore, our results suggest that cryptic refugia might have contributed to post glacial population expansion. These results have implications for studies of migration, estimation of range shifts and the role of microclimates under changing conditions. As new markers with greater resolution are currently developed (e.g. SNP based on a large panel of transcriptome variants), it may be possible in the near future to add more evidences of specific location from northern refugia of Scots pine populations and its role in subsequent migrations.

2.2Introduction

Migrations patterns of tree species in response to past climate changes have been a subject of increased debate during the last decades (Comes & Kadereit 1998, Davis & Shaw 2001, Cornille et al. 2013, Souto et al. 2015). Traditionally, it had become widely accepted that, in Europe, tree species retreated to the Iberian, Italian or Balkan peninsulas (Bennett et al. 1991, Petit et al., 2003) during the Last Glacial Maximum (LGM, around 26,000 - 19,000 yrs (Chiverrell & Thomas 2010)) and expanded North as the ice sheet melted during the Holocene warming. However this is likely to have been an oversimplification as environmental heterogeneity and geographic complexity might have created complex species persistence and migration scenarios. Although a large part of the Northern hemisphere was covered by ice with temperatures 10-20°C cooler than the present (Barron & Pollard 2002), increasing amounts of evidences suggest that areas located north of the peninsular refugia might have been suitable for some sparse populations to survive (Willis et al. 2000, Magri et al. 2006, Bhagwat & Willis 2008, Svenning et al. 2008, Birks & Willis 2010, Parducci et al. 2012, Tzedakis et al. 2013, Daneck 2016). Where these refugial areas were located continuous to excite scientific interest, as both the location of glacial refugia and patterns of subsequent migrations might have shaped the present day distribution of trees and therefore influenced current patterns of genetic diversity (Hewitt 2000). Understanding the structure and mode of the postglacial

colonization of Europe by trees is of particular interest for improving predictions of likely range shifts and adaptation of tree species in response to climate change.

Scots pine is one of the most widespread tree species in the world and has high ecological and economic value. Several studies based on fossil, pollen or genetic data, have investigated the existence of northern glacial refugia and postglacial migrations of this species and suggest that not only southern but also northern refugia might have contributed to contemporary patterns of European genetic diversity of Scots pine (Stewart & Lister 2001, Cheddadi et al. 2006, Donnelly et al. 2016). Several areas have been proposed to have harboured sparse populations of Scots pine during the LGM, such as the Eastern Alps, Hungarian plain and Danube region (Willis et al. 2000, Willis & van Andel 2004, Cheddadi et al. 2006), Eastern Carpathians (Tóth et al. 2017), Asia Minor (Naydenov et al. 2007), southern Scandinavia and other sites at mid-European latitudes (Pyhäjärvi et al. 2008, Prus-Glowacki et al. 2012, Parducci et al. 2012, Dering et al. 2017) or even southern of Russia (i.e. south east of Moscow) (Buchovska et al. 2013). Persistence of the species in small northern refugia would have significant implications for subsequent patterns of colonization, and more rigorous testing of hypothesis on the location of glacial refugia, Holocene migration routes and associated demographic events is needed.

According to the most recent studies, the likely pathways of Scots pine Holocene migrations in Europe would have crossed the continent in several fronts. In Western

Europe, Scots pine would have migrated north via the English Channel into Britain and via Danish Straits into Norway and the source of this front might have been located in dispersed central European or Balkan populations (i.e. considering the Balkan region delimited by the Danube river limit approximately) (Pyhäjärvi et al. 2008, Dering et al. 2017, Tóth et al. 2017). In Eastern Europe, Scots pine would have migrated towards Eastern Fennoscandinavia (i.e. Finland) and Central Europe from a refugia located somewhere in the NW Russian Plain (Buchovska et al. 2013, Tzedakis et al. 2013) or from the Ural Mountains (Naydenov et al. 2007). Iberian, Italian and Asia Minor peninsulas would have harboured Scots pine populations during the LGM, however they might have play no role in the subsequent Holocene colonization (Naydenov et al. 2007, Pyhäjärvi et al. 2008, Donnelly et al. 2016, Piotti et al. 2017).

An outstanding question remains regarding the origins of Scots pine populations in Scotland (UK), for which multiple colonization sources have been suggested. Pollen data have shown that Scots pine reached southern Britain via central Europe after the LGM, approximately 9000 years ago (Birks 1989) and from there colonized northward. However, pollen data indicated an independent glacial refugia in 8500 years ago well ahead of the main colonizing front likely located in the south of Britain by that time (Birks 1989, Bennett 1984, 1995). Fossil pine stomata recorded in western Scotland up to 1600 yrs ago prior to the arrival times indicated by traditional palynological methods, support this finding (Froyd 2006). Additional

studies have added evidence in support of the multiple centre of origin idea, such as a unique mitotype (Sinclair et al. 1998), unique cpDNA variation (Provan et al. 1998), and significant differences among monoterpene composition in western Scotland when comparing them with other populations from Scotland (Forrest 1980, Kinloch et al. 1986), although none have been conclusive. Alternative hypotheses have been proposed to explain the second origin, as due to colonization via a Westerly route originating in Southern Europe (Ennos et al. 1997) or the existence of an endemic Western refugium in the Western Isles (Kinloch et al. 1986), perhaps on now-submerged continental shelf, or in the south of Britain (Birks 1989). Recently, Donnelly et al. (2016) found that Scottish populations of Scots pine were highly related to Polish populations by the study of new variants of the mitochondrial genome, and Wójkiewicz & Wachowiak (2016) and Wójkiewicz, Litkowiec, et al. (2016) found similarities with populations from Central Europe but also with the north of the Iberian Peninsula and the Massif Central in France.

Phylogeographic approaches based on genetic information can be used to search for common ancestors (i.e. lineages) and to help to identify patterns of recolonisation during expansion phases (Provan & Bennett 2008). Although pollen and fossil data are the strongest indications of the location of a species in the past, hindcasting using species distribution models (SDM) based on inferences of past climate data can increase resolution by identifying potentially suitable areas even in the absence of physical evidence. Complementary use of phylogeographic reconstructions and

SDM, can deliver a robust analysis of past colonization ranges and the putative locations of refugial populations (Knowles & Alvarado-Serrano 2010). For instance, this novel combined approach has been successfully used to identify the direction of past European migrations of English yew (*Taxus baccata*) (Mayol et al. 2015) and to investigate the contribution of alternative refugia and postglacial migrations in the southernmost silver fir (*Abies alba*) populations (Piotti et al. 2017).

In this study we combined genetic and palaeoenviromental data to assess how past demographic processes have led to the present day distribution of genetic diversity of Scots pine at the European scale and, in particular, to better understand the unresolved origins of the North-Western populations of Scots pine. Specifically we sought to determine: (1) the differences between northern populations and peninsular refugial populations of Scots pine in Europe, (2) the role of peninsular refugial populations to subsequent Holocene migration and main sources for central and northern European populations, and (3) the contribution of single or multiple sources that originate North-Western populations of Scots pine which have been subject to much debate in the literature. Our results help to further disentangle the demographic history of Scots pine and the controversial origin of the North-Western populations.

2.3 Material and methods

2.3.1 Genetic datasets and markers

We examined nuclear (nSSR) and chloroplast microsatellite markers (cpSSR) and single nucleotide polymorphism (SNP) data from populations across Europe (European SSR and European SNP hereafter, see Fig. 2.1 and supplementary material S2.1) (Wachowiak et al. 2011, Wójkiewicz & Wachowiak 2016, Wójkiewicz et al. 2016) to estimate genetic diversity and population structure, and to test for likely source populations and subsequent routes of colonization. Additionally, we used SSR data from approximately 25% of all remaining indigenous populations in Scotland (both nSSR and cpSSR) (Scottish SSR, hereafter, see Fig 2.1) (González-Díaz et al., in review) to test for likely sources, population size changes and patterns of assembly. The use of multiple genetic data can provide additional interpretations of demographic events than either alone. Although each marker type has a different mode of evolution, concordance in patterns inferred from them secures the inference against bias due to data type. All markers were used for understanding current spatial distribution of genetic diversity at European scale, but only the nuclear ones (nSSR and SNP) for subsequent historical demographic modelling.

Thirteen European nSSR were obtained from 637 individuals and thirteen European cpSSR from 633 individuals, both from 23 sites along the continent (Fig. 2.1, green dots). Specifically we used nSSR: psyl2, psyl18, psyl25, psyl36, psyl42, psyl44, psyl57 (Sebastiani et al. 2011), SPAC7.14, SPAC11.4 (Soranzo et al. 1998), PtTX2146, PtTX3107, PtTX3025 and PtTX4011 (Aukland et al., 2002); and cpSSR: PCP1289, PCP26106, PCP30277, PCP36567, PCP41131, PCP45071, PCP87314, PCP102652

(Provan et al. 1998), Pt15169, Pt26081, Pt36480 and Pt71936 (Vendramin et al., 1996) (see Wójkiewicz et al. (2016) for nSSR genotyping protocol and Wójkiewicz & Wachowiak (2016) for cpSSR protocol). In addition we used a dataset comprising 1650 haplotypic European SNPs from 135 individuals from 14 populations (Fig. 2.1, red dots) (See Wachowiak et al. (2011) for SNP genotyping protocol).

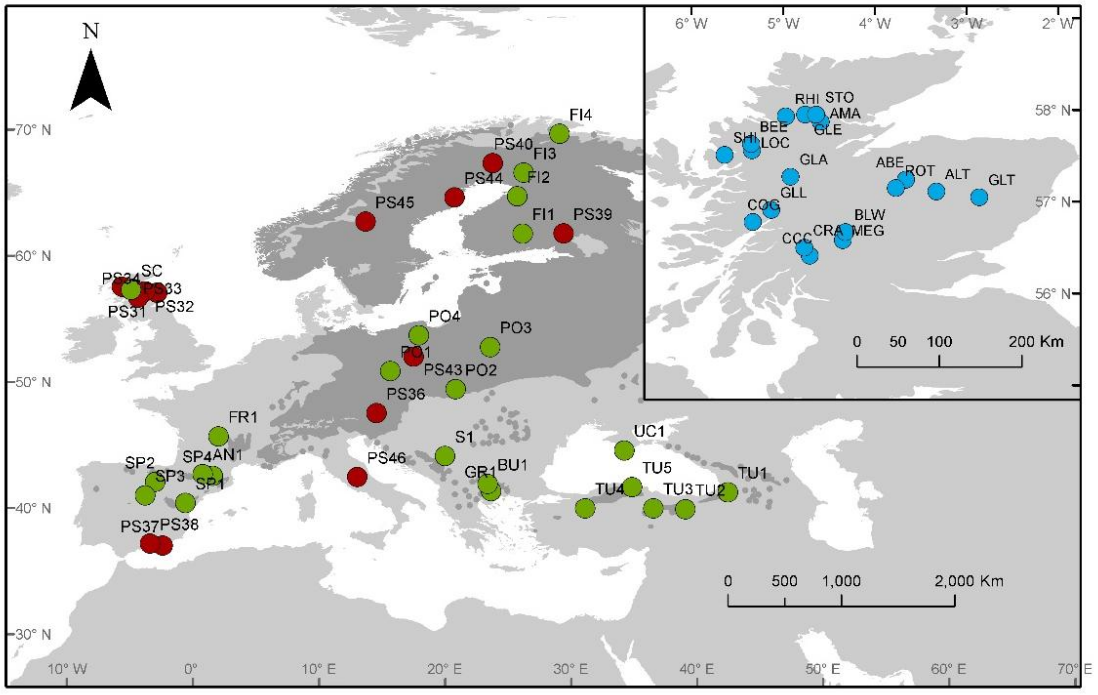

Figure 2.1: Study sites for European nuclear and chloroplast microsatellite markers (European SSR, green dots) and single nucleotide polymorphism markers (European SNP, red dots), and the study sites for Scottish nuclear and chloroplast SSR markers (Scottish SSR, blue dots). Shaded area represents contemporary Scots pine distribution (EUFORGEN, http://www.euforgen.org/species/). See supplementary material S2.1 for further description of the European SSR sites.

For Scotland-focussed analysis, we used a dataset comprising six Scottish nSSR and five Scottish cpSSR from 540 individuals from 18 populations across Scotland (Fig. 2.1, blue dots). Specifically the nSSR loci were: PSAJ223770/SPAC11.14, PSAJ223766/SPAC11.8 (Soranzo et al. 1998), Ptx2146 (Aukland et al. 2002),

SsrPt_ctg4698, SsrPt_ctg9249 (Chagné et al. 2004) and LOP3 (Liewlaksaneeyanawin et al. 2004); and cpSSR, PCP26106, PCP30277, PCP36567, PCP45071, PCP87314 (Provan et al. 1998) (see González-Díaz et al. (in review) for microsatellite analysis specification).

2.3.2 Population structure and spatial patterns of genetic diversity at European scale

To measure genetic diversity at the European scale we calculated genetic estimators for nSSR and cpSSR, within and among populations and regions using FSTAT 2.9.3.2 (Goudet 1995) and Arlequin v3.5 (Excoffier & Lischer 2010). Regions were determined according to the results of STRUCTURE which supported three genetic clusters (K=3) for both datasets (Fig. 2.6) and highly admixed populations as distinct groups (see section 2.4.1 for detailed information about the regions). Specifically for nSSR, we estimated mean number of alleles per locus (*nA*), rarefied allelic richness (nA_R), observed heterozygosity (nH_O), expected heterozygosity (nH_E) and inbreeding coefficient (nF_{IS}). Rarefaction of allelic richness controls for differences in sample size (El Mousadik & Petit 1996) allowing comparison among sites and studies. For the remaining cpSSR estimators, alleles were combined to create a unique chloroplast haplotype for each individual. We estimated number of haplotypes (cH_N), number of private haplotypes (cH_P) and gene diversity corrected for sample size (cH_E) (Nei, 1978). Genetic diversity estimators for SNP data within and among genetic clusters

were estimated using DnaSP (Librado & Rozas 2006). We calculated the number of haplotypes (sH_N), number of polymorphic sites (S), number of mutations (M), nucleotide diversity (π), Tajima´s D (D), Theta per site (T) and haplotype diversity (sH_E). Multilocus haplotypic and neutral diversity of individual populations for nSSR (nH_E), cpSSR (cH_E) and SNP (π) were mapped using ARCMAP 10 (ESRI, Redlands, CA, USA).

To quantify genetic differentiation we conducted permutation tests (10,000 permutations) in FSTAT in nA_R, nH_O, nH_E and nF_{IS} between regions. Additionally we used both nSSR and cpSSR markers in Arlequin v3.5 (Excoffier & Lischer 2010) to estimate nF_{ST} and cF_{ST} among regions, using nSSR and cpSSR respectively and to assess the distribution of variation. xF_{ST} is used as a measure of population differentiation by estimating differences in allele frequencies. Significance values were determined for a 5% nominal level after Bonferonni correction. To quantify the distribution of variation of nuclear genetic diversity and chloroplast haplotypes, we tested both markers in a hierarchical analysis of molecular variance (AMOVA) from the level of individual, population and region.

To determine population structure, an individual-based Bayesian assignment method was used using data from nuclear loci (nSSR and SNP) in STRUCTURE 2.3.4. (Pritchard et al. 2000). We used a model with correlated allele frequencies (Falush et al. 2003) and admixtured ancestry. We included the site location *a priori* (LOCPRIOR option) to improve the detection of weak population structure (Hubisz

et al. 2009). *K*, which is the more likely number of genetic clusters, was set from 1 to 20, with 10 runs performed for each number of *K*. Runs consisted of 500,000 Markov Chain Monte Carlo (MCMC) iterations with a burn-in period of 100,000. We used STRUCTURE HARVESTER (Earl & VonHoldt 2011), an online application that uses the Evanno method (Evanno et al. 2005) for assessing and visualizing likelihood values across multiple values of *K* and detecting the number of genetic groups that best fit the data.

2.3.3 Analysis of demographic history

We used an approximate Bayesian computation (ABC) framework implemented in DIYABC v2.1.0 (Cornuet et al. 2014) to explore scenarios of demographic history that were likely to have generated current genetic structure of Scots pine across the continent and in Scotland only, according to the results obtained from analyses of genetic structure. We carried out two sets of demographic analyses using nuclear markers. The first analysis aimed to identify source populations (i.e. likely glacial refugia) and migration routes at the continental scale. For these analyses we used European SSR (nSSR) and European SNP data (see Fig. 2.1, red and green dots). The second analysis explored changes in population size during and since colonization at a finer scale in Scotland and we used Scottish SSR (nSSR) data (Fig. 2.1, blue dots).

2.3.3.1 Identification of glacial refugia and migration routes

We followed a two step approach within each dataset. The first step, which included models 1 & 3 (see Table 2.1), aimed to identify putative refugia and source populations of Central-Northern European population; and the second step, which included models 2 & 4 (see Table 2.1 and Fig. 2.2&2.3), aimed to identify the source of Scottish populations. For the second step, we compared the two scenarios that showed comparably high posterior probability in the first step 1.

Table 2.1. A graphical overview of the two step approach for each dataset (European nSSR and SNP) used for the identification of glacial refugia, migration patterns and source of colonization in Central-Northern European populations (step 1, model 1 & 3) and Scottish populations (step 2, model 2 & 4). Population names are coded as follows: Italy (ITA), Iberia (IBE), Balkan (BAL), Asia Minor (AMI), Central-Northern Europe (CNE), Scotland (SCO) and ancestral population (ANC).

Dataset	Step and model	Scenario	Glacial refugia and source of colonization of Central-Northern Europe population (step 1)	Source of colonization of Scotland (step 2)
European nSSR	Step 1, model 1	1a	IBE	
		1b	BAL	
		1c	AMI	
		1d	Admixture IBE-BAL	
		1e	Admixture BAL-AMI	
		1f	Established previous to the LGM	
	Step 2, model 2	2a	Admixture IBE-BAL	Admixture IBE-CNE
		2b	Admixture IBE-BAL	CNE
		2c	Admixture IBE-BAL	IBE
		2d	BAL	Admixture IBE-CNE
		2e	BAL	CNE
		2f	BAL	IBE
European SNP	Step 1, model 3	3a	IBE	
		3b	ITA	
		3c	Admixture IBE-ITA	
		3d	Established previous to the LGM	
	Step 2, model 4	4a	IBE	CNE
		4b	IBE	IBE
		4c	IBE	Admixture IBE-CNE
		4d	Established previous to the LGM	CNE
		4e	Established previous to the LGM	Iberian
		4f	Established previous to the LGM	Admixture IBE-CNE

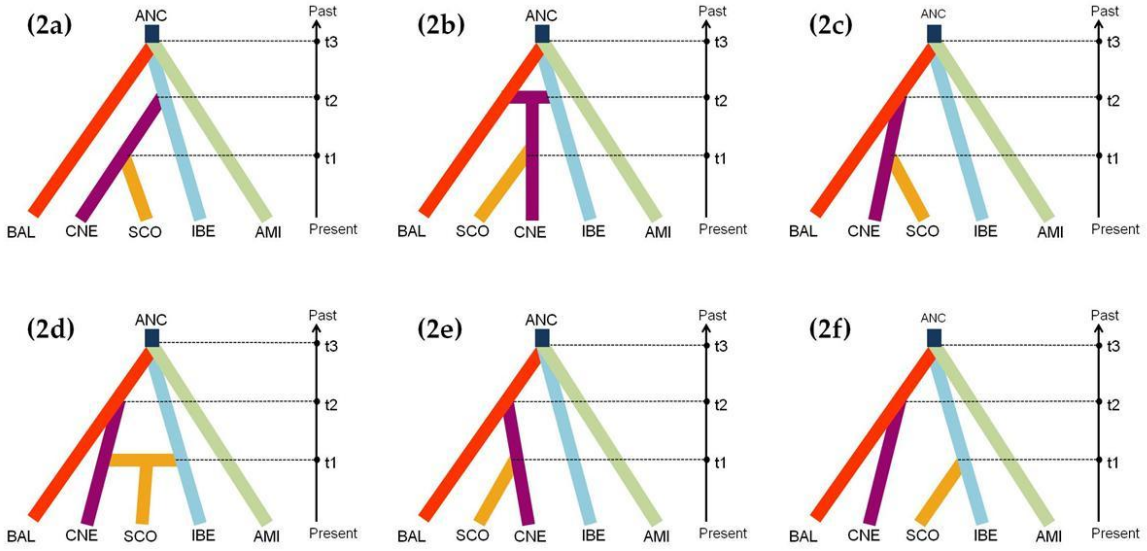

Figure 2.2 Colonization scenarios of Scots pine tested using the European nSSR data. Population names correspond to Iberia (IBE), Balkan (BAL), Asia Minor (AMI), Central-Northern Europe (CNE), Scotland (SCO) and ancestral population (ANC). *t1*, *t2* and *t3* correspond to the divergence times in generations. More details on each scenario (indicated by a label) can be found in Table 1; Step2, model 2 section.

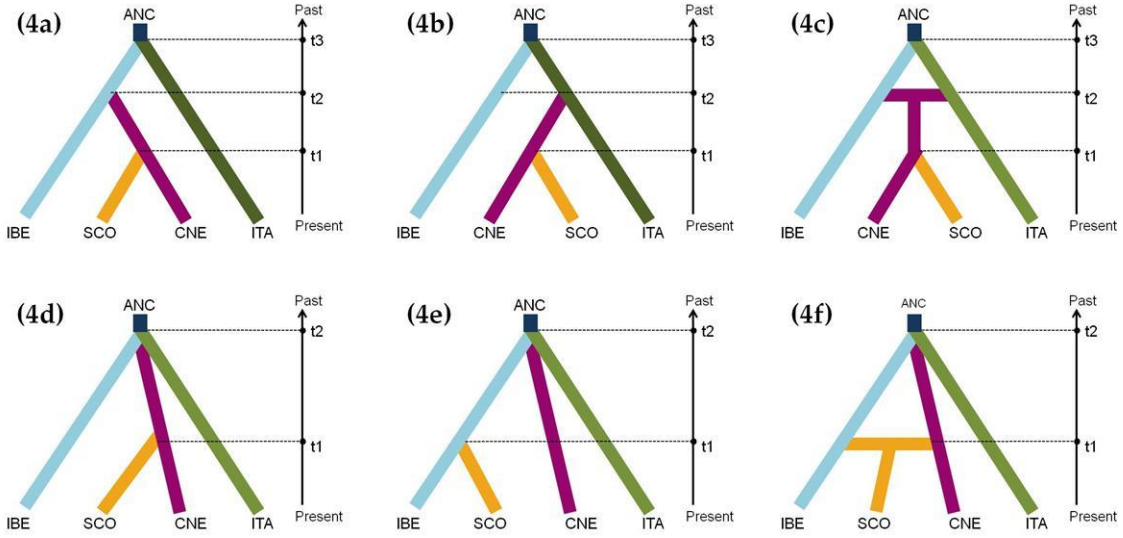

Figure 2.3 Colonization scenarios of Scots pine tested using the European SNP data. Population names correspond to Italy (ITA), Iberia (IBE), Central-Northern Europe (CNE), Scotland (SCO) and ancestral population (ANC). *t1*, *t2* and *t3* correspond to the divergence times in generations. More details on each scenario (indicated by a label) can be found in Table 1; Step2, model 2 section.

Prior parameters for effective population sizes (N_X) and timing of events (t_X) were defined based on knowledge of Scots pine colonization dynamics from palynological, historical and genetic information. Prior parameter distributions for the SSR model were uniform and bounded between 10-15×10^4 for the effective population size for Asia Minor (N_{AMI}), Balkans (N_{BAL}), Iberia (N_{IBE}), Central-Northern Europe (N_{MEU}), Scotland (N_{SCO}) and the ancestral population (N_{ANC}). Population divergence time priors ranged between 10-5000 generations for *t1*, 10-10×10^3 for *t2* and *t3*, and 10-10×10^4 for *t4*, with the additional setting *t4>t3*, *t3>t2*, and *t2>t1*. Prior parameter distributions for the SNP model were also uniform and ranged between 10-10×10^4 for the effective population size for Italy (N_{ITA}), N_{IBE}, N_{SCO}, and N_{ANC}, and between 10-10×10^5 for N_{MEU}. Population divergence time priors ranged between 10-5000 for *t1*, 10-10×10^4 for *t2, t3* and *t4*, with the additional setting *t4>t3*, *t3>t2*, and *t2> t1*. Priors for admixture rates for both SSR and SNP models ranged between 0.01 and 0.99.

Nuclear microsatellite loci (nSSR) were assumed to follow a Generalized Stepwise Mutation model and a uniform prior was assumed for the mean microsatellite mutation rate bounded between 10^{-3} and 10^{-4}. Thirty-six summary statistics were used for the ABC analysis. Three single sample statistics were used (mean number of alleles, mean Nei's genetic diversity index (Nei, 1987) and mean allele size variance), and three between-sample statistics (mean allele size variance, F_{ST}, and mean index of classification (Rannala & Mountain 1997, Pascual et al. 2007). For the SNP data

four one-sample statistics were used (proportion of zero values, mean of non-zero values, variance of non-zero values and mean of complete distribution). Additionally, four two-sample statistics were also used (F_{ST} distances based on the proportion of zero values and the variance of non-zero values, and Nei´s distances based on the proportion of zero values and the variance of non-zero values). Following the methods outlined by Cornuet et al. (2010), type I and type II errors were estimated to evaluate the power in both SSR and SNP models. Type I error (wrongly accepting false scenario) was computed for as the proportion of simulated scenarios generated under the focal scenario that support other scenarios, and type II error (wrongly rejecting true scenario) as the proportion of datasets simulated under all other scenarios that was assigned to the focal scenario (Lepais et al., 2013). The most favoured scenario was used to estimate posterior distribution of demographic parameters.

2.3.3.2 Changes in population size

In addition, we performed a demographic history analysis of the Scottish population using the scottish SSR (nSSR) data to determine changes in population size during and since colonisation. We considered Scottish populations as a single unstructured group. Four scenarios were tested (Fig. 2.4): the first scenario assumed no change in population size (constant); the second assumed a population expansion; the third assumed a decline in the populations and the last assumed a bottleneck in

population size followed by re-expansion. Priors for effective population sizes and timing of events were defined based on knowledge of Scots pine colonisation dynamics from palynological and historical information. Prior parameter distributions were uniform and bounded between 10-5x10^5 for the effective population sizes. Time priors ranged between 10-700 for the duration of bottleneck (*db*) and 10-2000 yrs for *t2*, with the additional setting *t3>t2*, and *t2>t1*. Four single sample summary statistics were used for the ABC analysis (mean number of alleles, mean Nei's genetic diversity index (Nei, 1987), mean allele size variance and mean Garza and Williamson's M). Type I and type II errors were estimated to evaluate the power of the model. The most favoured scenario was used to estimate posterior distribution of demographic parameters.

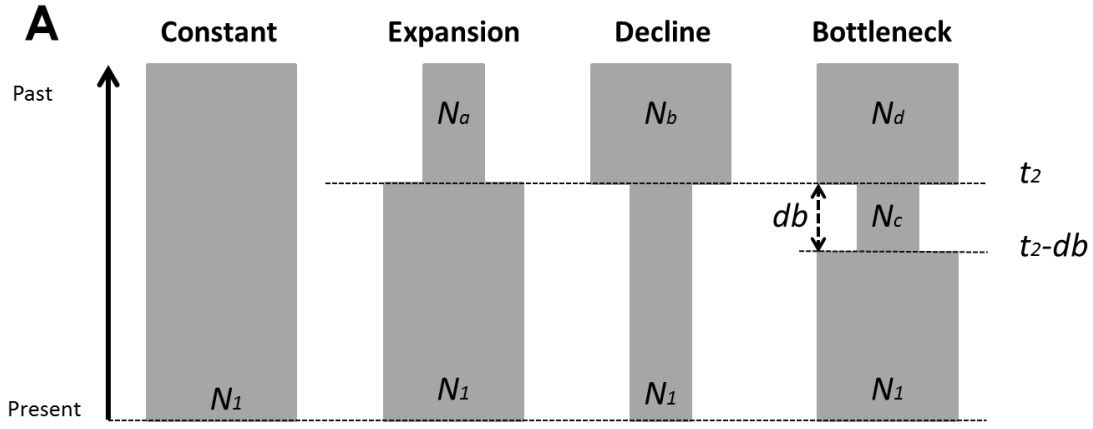

Figure 2.4 Scenarios of changes in population size of Scots pine tested, that assume (i) constant (i.e. no change in population size), (ii) expansion, (iii) decline, or (iv) bottleneck . Parameters N_1, N_a, N_b, N_c and N_d refers to effective population sizes, t_2 is the time in generations at which the change in population size occurred and *db* is the duration of the bottleneck.

2.3.4 Species distribution modelling

The distribution of Scots pine for the present, the Last Glacial Maximum (LGM) and the Last Interglacial period (LIG) was estimated. Scots pine occurrence data were obtained from three different sources (see supplementary material S2.3): (1) the EUFORGEN database from the European forest genetic resources network (http://www.euforgen.org/distribution _maps.html), (2) the Tree Species Distribution for Europe (TSDE; (Köble & Seufert 2001)) from the Joint Research Centre's AFOLU data portal (http://forest.jrc.ec.europa.eu/activities/climate-change/species-distribution/), and (3) the European National Forest Inventory (NFI) data. EUFORGEN delivers a distribution shapefile that, while spatially less accurate, includes the whole species range within Europe, and excludes planted populations. On the other hand, TSDE maps tree species occurrence in Europe at a 1-km grid level (30 arc sec.), but it does not differentiate between native and planted populations. Therefore, by filtering TSDE occurrences with EUFORGEN, we obtained a good approximation of the native range of species (Serra-Varela et al. 2015). For the combination of TSDE and EUFORGEN occurrence data, a total of 23,840 presences were used for the calibration of the model at 2.5 min resolution. As recommended by Barbet-Massin et al. (2012) a large number of pseudo-absences were used. Specifically we used 10,000 pseudo-absences randomly selected within the study area within those cells where TSDE data reported 0% occupancy. Regarding the

occurrence data from the European inventory data, which provides information on adult individuals and regeneration, we used a total of 3,998 presences for the adult data and a total of 243 presences for regeneration (seedlings) again at 2.5 min resolution. We used 2000 pseudo-absences for adults and 200 for regeneration.

Firstly, we performed a variable selection over the bioclim dataset of 19 climatic variables (See supplementary material S2.2). As the variables were highly correlated we applied a principal components analysis (PCA) and correlation coefficients on all 19 highly correlated climatic predictors, but also taking into account those biological factors limiting the growth and regeneration of the species (i.e. low water availability). We used the resultant selection of variables under current and past conditions (paleoenvironmental data), the latter representing conditions at the Last Glacial Maximum (LGM) and the Last Interglacial period (LIG). For the LGM period, we used both the CCSM and MIROC General Circulation Models (GCMs).

Five statistical algorithms were used to calibrate the species distribution models individually. Specifically, we used general linear models (GLMs; McCullagh & Nelder, 1989), generalized additive models (GAMs; Hastie & Tibshirani, 1990), random forest (RF; (Breiman 2001)), classification tree analysis (CTA; Breiman et al., 1984) and MaxEnt (Phillips et al. 2006). All models were processed using the package 'biomod2' (Thuiller et al. 2009) in R 3.3.1 (R Development Core Team 2011). The performance of the model was assessed by means of the true skill statistic (TSS) and the area under the receiver operating characteristic curve (ROC). We averaged the

predictions from the five individual algorithms to create an ensemble model (e.g. refs). We evaluated the model on 20% of the set-aside evaluation records.

2.4 Results

2.4.1 Genetic diversity and population structure at European scale

Across the thirteen nuclear loci (nSSR) analysed for the continental sample, the number of alleles per locus (*nA*) ranged from 3 to 40, with a multilocus average of 12.231 ± 10.256 for all populations combined. For rarefied allelic richness (nA_R), multilocus estimates ranged from values of 4.565 to 6.176, based on a minimum number of 19 diploid individuals, with a multilocus average of 12.211 for all populations combined (based on minimum number of 651 individuals) (see Table 2.2 for detailed information of each population and for each region). The multilocus expected (nH_E) and observed heterozygosity (nH_O) were 0.527 ± 0.298 and 0.446 ± 0.267 respectively for all populations combined (Table 2.2). A general significant homozygote excess was found (overall F_{IS}=0.154, $P < 0.001$) (Table 2.2). Among the thirteen chloroplast loci (cpSSR) analysed in the continental sample, the number of alleles per locus (c*A*) ranged from 3 to 11, with a multilocus average of 6.308 ± 2.359 for all populations combined. Gene diversity (cH_E) ranged between 0.970 and 1, with a slight increase of diversity towards the north (Table 2.2, Fig. 2.5a). The number of haplotypes (cH_N) within populations ranged from 19 to 42 (Table 2.2 and Fig. 2.5b), with 468 haplotypes recorded, of which 73% (342 haplotypes) were private (i.e.

unique of a particular population, Table 2.2). In the European SNP dataset, there were 135 haplotypes and 510 polymorphic sites, with an overall nucleotide diversity, Pi: 0.08762 when all sites were combined (Table 2.3 and Fig. 2.5c). Tajima's D for all samples combined was -1.62047 with no statistical significance using the total number of mutations and -1.52635 with no statistical significance using the total number of segregating sites, with an estimated recombination rate R, between adjacent sites of 0.0271 (excluding sites with gaps or missing data) (Table 2.3). When populations were grouped into genetic clusters, significant differences in nA_R and nH_E (p-values < 0.05) occurred between genetic clusters after 10,000 permutations, but not in nH_O and F_{IS} (p-values > 0.05).

After accounting for the population structure the most likely number of clusters identified, for both nSSR and SNP markers, was three (Fig. 2.6). Populations from Iberia, Asia Minor and Italy constituted independent genetic clusters, and Scotland and Balkan populations as highly admixed populations. Regions were determined according to the population structure results which supported three genetic clusters (K=3) for both datasets (Fig. 2.6) and highly admixed populations as distinct groups. In total, for the SSR dataset we considered five regions (Iberian, Balkan Peninsula, Asia Minor, Central-Northern Europe and Scotland, Fig. 2.2) and for the SNP dataset we considered four regions (Iberian, Italian peninsula, Central-Northern Europe and Scotland, Fig. 2.3).

Distribution of SSR (nSSR and cpSSR) variation from the AMOVA results showed that although most of the variation in the continental sample was found within populations, among cluster variability was greater and more significant than among population within cluster (Table 2.4). F_{ST} estimates from European SSR data showed Asia Minor to be the most differentiated (greatest F_{ST} values for both nSSR and cpSSR), whereas the lowest differentiation occurred among the Balkans and Central-Northern Europe (i.e. although significant, nF_{ST} was one order of magnitude lower than other pairwise comparisons: cF_{ST} was not significant and again one order of magnitude lower, Table 2.5).

Across the six nuclear loci (nSSR) analysed for the Scottish sample, the multilocus estimates for rarefied allelic richness (nA_R) ranged from values of 5.42 ± 2.92 to 7.55 ± 3.15, based on a minimum number of 28 diploid individuals, with a multilocus average of 5.84 for all populations combined. The multilocus expected (nH_E) and observed heterozygosity (nH_O) ranged from 0.46 ± 0.21 to 0.61 ± 0.06 and from 0.51 ± 0.27 to 0.66 ± 0.16 respectively. Among the five chloroplast loci (cSSR) analysed, gene diversity (cH_E) ranged between 0.83 and 0.96 and the number of haplotypes (cH_N) within populations ranged from 13 to 19, with a total of 64 haplotypes recorded, of which 42% (27 haplotypes) were private (unique to a particular population) (see Chapter 3, Table 3.3 for more detailed information about Scottish nSSR and cSSR)

Table 2.2 Genetic estimators for Europe *nSSR* and *cpSSR* for each population and region. nA_R was rarefied for 19 individuals at the population level and for 38 individuals at the regional level.

Population/ Region	*nSSR*										*cpSSR*			
	nN	*nA*		nA_R		nH_O		nH_E		nF_{IS}	*cN*	cH_N	cH_P	cH_E
Turkey1	25	5.462	(4.013)	5.185	(3.672)	0.477	(0.297)	0.525	(0.302)	0.094**	25	23	10	0.993
Turkey2	25	5.769	(4.146)	5.374	(3.716)	0.423	(0.288)	0.510	(0.324)	0.175***	25	24	15	0.997
Turkey3	25	5.538	(3.178)	5.338	(3.001)	0.456	(0.259)	0.518	(0.282)	0.122***	25	21	10	0.983
Turkey4	25	5.769	(4.438)	5.479	(4.053)	0.418	(0.276)	0.513	(0.276)	0.189***	25	23	9	0.993
Turkey5	25	6.231	(5.134)	5.828	(4.544)	0.483	(0.249)	0.532	(0.259)	0.094**	25	19	8	0.977
Ukraine	25	4.692	(3.966)	4.565	(3.736)	0.467	(0.290)	0.507	(0.287)	0.080*	25	19	8	0.970
Greece	31	6.615	(5.173)	5.858	(4.073)	0.494	(0.273)	0.544	(0.286)	0.094***	31	28	13	0.994
Bulgaria	25	6.308	(4.131)	5.876	(3.933)	0.490	(0.272)	0.540	(0.294)	0.095**	25	22	13	0.990
Serbia	26	6.154	(4.120)	5.680	(3.836)	0.481	(0.283)	0.525	(0.307)	0.086**	26	24	17	0.991
Spain1	29	6.538	(5.093)	5.840	(4.167)	0.494	(0.261)	0.591	(0.230)	0.166***	25	22	7	0.990
Spain2	31	5.846	(5.194)	5.274	(4.233)	0.460	(0.279)	0.532	(0.276)	0.136***	31	29	19	0.996
Spain3	29	5.769	(4.850)	5.260	(4.106)	0.452	(0.288)	0.509	(0.282)	0.113***	29	25	11	0.990
Spain4	32	5.923	(5.008)	5.318	(4.139)	0.494	(0.300)	0.530	(0.278)	0.068***	32	30	20	0.996
Andorra	32	6.308	(4.956)	5.529	(4.063)	0.452	(0.265)	0.538	(0.284)	0.161*	32	32	16	1.000
France	25	6.308	(4.956)	5.934	(4.392)	0.481	(0.293)	0.539	(0.273)	0.109***	25	23	15	0.993
Poland1	33	7.077	(6.357)	6.176	(5.132)	0.406	(0.246)	0.566	(0.273)	0.286***	33	30	15	0.994
Poland2	45	7.000	(6.110)	5.663	(4.666)	0.470	(0.242)	0.553	(0.278)	0.151***	45	42	25	0.996
Poland3	22	5.462	(4.977)	5.281	(4.695)	0.401	(0.289)	0.486	(0.342)	0.179***	22	22	16	1.000
Poland4	28	6.231	(5.418)	5.655	(4.808)	0.443	(0.253)	0.524	(0.308)	0.156***	28	28	16	1.000
Finland1	25	6.385	(5.546)	5.793	(4.875)	0.510	(0.312)	0.540	(0.301)	0.057	25	24	15	0.997
Finland2	25	6.385	(5.268)	5.854	(4.757)	0.491	(0.335)	0.500	(0.321)	0.019	25	25	13	1.000
Finland3	25	5.923	(4.804)	5.436	(4.161)	0.477	(0.254)	0.539	(0.296)	0.117***	25	24	11	0.997
Finland4	24	5.769	(4.512)	5.427	(4.127)	0.497	(0.278)	0.520	(0.297)	0.046	24	23	15	0.996
Scotland	39	6.750	(4.751)	5.342	(3.760)	0.473	(0.226)	0.538	(0.237)	0.123***	39	39	25	1.000
Asia Minor	125	8.538	(6.691)	7.136	(5.249)	0.452	(0.259)	0.534	(0.290)	0.153	125	94	52	0.9932

Balkan peninsula	107	8.846	(7.636)	7.347	(5.794)	0.485	(0.263)	0.536	(0.292)	0.096	107	91	51	0.9963
Iberian peninsula	178	9.692	(8.430)	7.147	(5.504)	0.442	(0.282)	0.515	(0.286)	0.143	174	134	88	0.9961
Central-Northern EU	227	10.385	(9.474)	7.375	(6.341)	0.430	(0.278)	0.501	(0.320)	0.142	227	183	126	0.9976
Scotland	39	6.750	(4.751)	6.284	(4.619)	0.473	(0.226)	0.538	(0.237)	0.123	39	39	25	1.000

Data from Table 2.2 include genetic estimators for (1) nSSR: *nN*, no. of samples; *nA*, no. of alleles; nA_R rarefied allelic richness; H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, inbreeding coefficient, and (2) cpSSR: c*N*, no. of samples; cH_N, no. of haplotypes; cH_P, no. of private haplotypes and cH_E, gene diversity corrected for sample size (Nei, 1978). Significant *P*-values are indicated as $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. *P*-values for F_{IS} were obtained after 1,000 permutations of gene copies within individuals of each stand. Standard errors are reported in brackets.

Table 2.3 Genetic estimators for Europe SNP data

Population	*sN*	sH_N	*S*	*M*	π	*D*	*T*	sH_E
Scotland1	10	10	227	230	0.07714	0.04289	0.07648	1
Scotland2	10	10	234	237	0.0793	-0.15359	0.08181	1
Scotland3	10	10	290	292	0.08758	0.40931	0.08096	1
Scotland4	10	10	217	217	0.07813	0.01621	0.07787	1
Scotland5	10	10	262	264	0.0907	0.23873	0.08657	1
Austria	10	10	320	321	0.08675	0.00382	0.08668	1
Spain1	10	10	278	280	0.07386	-0.06389	0.07481	1
Spain2	10	10	287	289	0.08604	0.16306	0.08333	1
Finland1	10	10	259	262	0.09543	-0.3065	0.10166	1
Finland2	10	10	105	106	0.06847	-0.04743	0.06913	1
Poland	10	10	141	141	0.08096	-0.35237	0.08714	1
Sweden1	8	8	205	207	0.08629	0.17054	0.08368	1
Sweden2	7	7	222	223	0.09583	0.14311	0.09355	1
Italy	10	10	88	88	0.04366	1.37144	0.03418	1

Data from Table 3 include genetic estimators for SNP data: *sN*, no. of samples; sH_N, no. of haplotypes; *S*, no. of polymorphic sites; *M*, no. of mutations; π, nucleotide diversity; *D*, Tajima´s *D* ; *T*, Theta per site; sH_E, haplotype diversity.

Table 2.4 Hierarchical analysis of molecular variance (AMOVA) for nuclear and chloroplast markers at the individual, population and region level (using five regions: three STRUCTURE clusters and two admixed clusters). The degrees of freedom (*df*), sum of squares (SS), percentage of variation explained by each level (Variation (%)), and the relevant *P*-values are indicated.

	nSSR				cpSSR			
Source of variation	*d.f.*	SS	Variation (%)	*p*-value	*d.f.*	SS	Variation (%)	*p*-value
Among regions	4	106.941	2.33	<0.001	4	26.525	1.02	<0.001
Among populations within regions	19	123.343	1.78	<0.001	19	57.227	0.34	0.115
Within populations	1328	4258.717	95.89	<0.001	648	1783.753	98.63	<0.001

Table 2.5 F_{ST} values for regions of nSSR (below the diagonal) and cpSSR (above the diagonal).

	Asia Minor	Balkans	Iberia	C-N Europe	Scotland
Asia Minor	-	0.00554	0.01443***	0.01016***	0.00354
Balkans	0.02334***	-	0.01875***	-0.00047	-0.00409
Iberia	0.05027***	0.02183***	-	0.02132***	0.00679
C-N Europe	0.04207 ***	0.00893***	0.02242***	-	-0.00153
Scotland	0.04634***	0.02138***	0.01360***	0.01605***	-

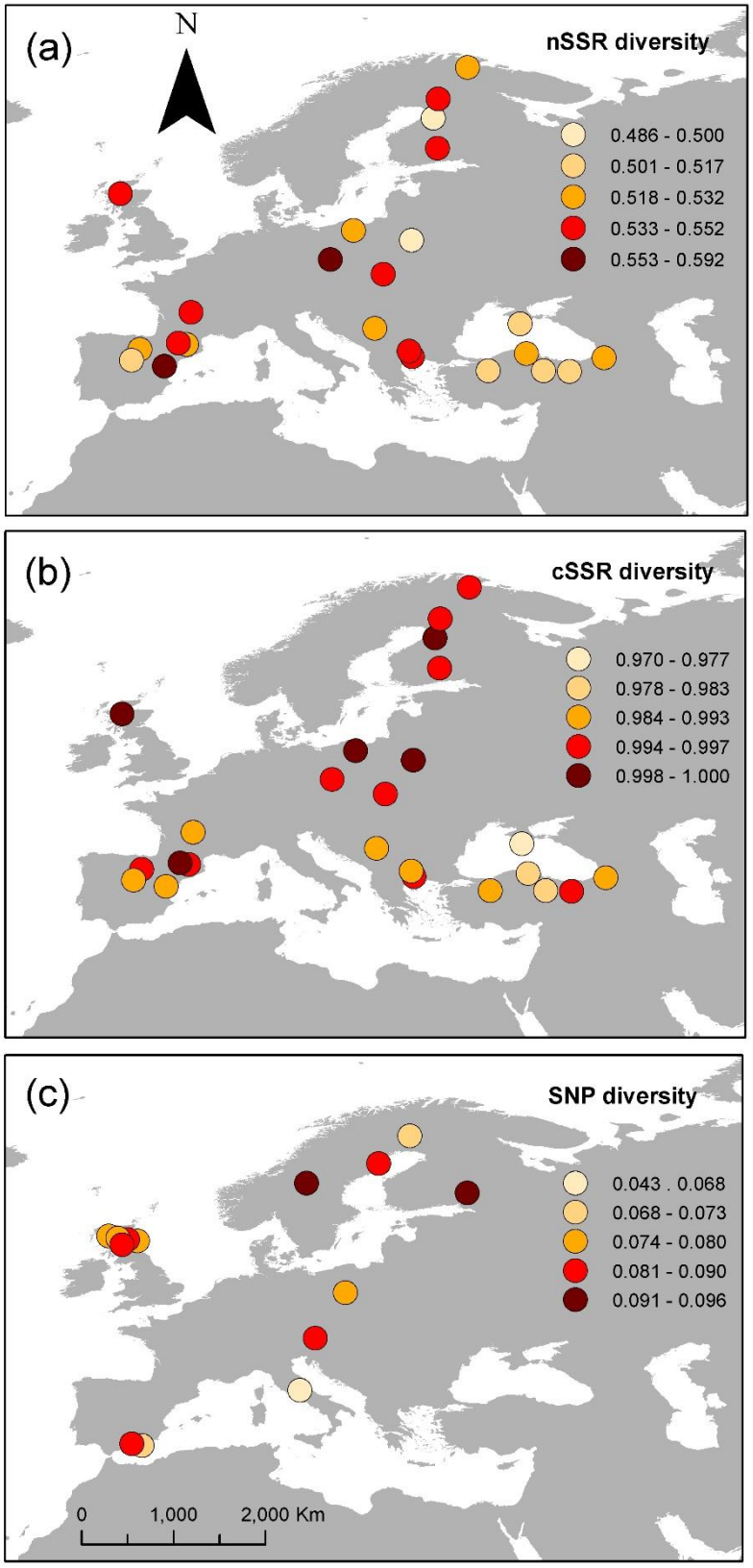

Figure 2.5 Diversity estimated using different markers: (a) nuclear (nSSR diversity), (b) chloroplast (cpSSR diversity), and (c) SNP (SNP diversity) for European populations of *Scots pine.*

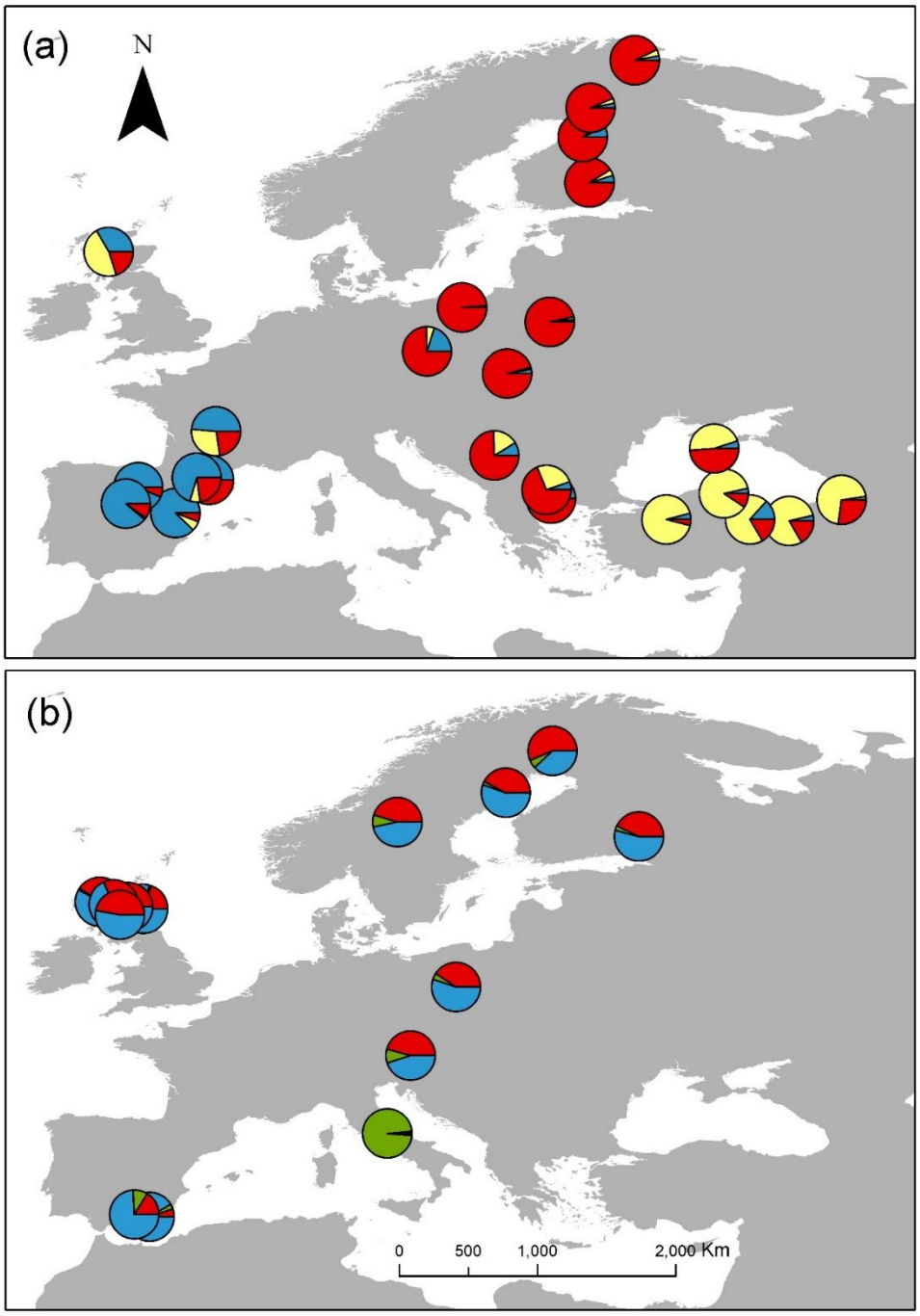

Figure 2.6 Optimal number of genetic clusters (K=3) obtained for (a) European nSSR and (b) European SNP datasets of Scots pine. Pie charts show the average values of 10 different runs for the proportion of membership to each genetic cluster. Different colours indicate different genetic clusters independently for each figure.

2.4.2 Demographic history

2.4.2.1 Identification of glacial refugia and colonization in Europe and Scotland

Model-based inference using ABC analyses for the nSSR dataset indicated that **scenario 1.2** (see Table 2.1), was supported with almost 60% probability compared to 30% of scenario 1.4 and almost no support for the other scenarios. This result suggest that *Scots pine* colonisation to the central-northern European sites comes at least partially from the Balkans and with lower probability from the Iberian Peninsula. In the second step, the **scenario 2.4** was supported with 60% probability compared to 30% of scenario 2.1, 2.2, 2.3, 2.5 and 2.6, which is an indicative of Scottish populations having arisen from admixture between colonising fronts originating in south western Europe (Iberian glacial refugia and/or southern France areas) and central-northern Europe (see Fig. 2.2 for step 2 scenarios of European SSR). The confidence in the best scenario is high, with low error rates in both Type I and Type II: (Table S3). For the scenario 2.4, median values of the effective population size were 67300, 112000, 101000, 1250000 and 47500 for *N1* (Asia Minor), *N2* (Balkan peninsula), *N3* (Spain and South France), *N4* (Central and Northern Europe) and *N5* (UK). The median values for divergence events corresponded to 673, 2600 and 6650 number of generations for *t2*, *t3* and *t4*, which could be translated into approximately 13460, 52000 and 133000, considering Scots pine reach maturity at the age of 20 years (Carlisle & Brown 1968). The rate of admixture (*ra*) corresponded to 0.430.

Model-based inference using ABC for the European SNP dataset indicated that **scenario 3.4** was supported with 80% compared to other scenarios, which indicate that central-northern European populations where established prior to the LGM and did not derive from Iberia or Italian. In the second step, **scenario 4.6** was supported with almost 100% probability compared to almost no support for other scenarios (see Fig. 2.3 for step 2 scenarios of European SNP). This result suggests that Scottish populations have arisen through admixture of colonisation fronts from south western Europe (i.e. Iberian glacial refugia or central massif of France) and central Europe. In addition, some central European populations might have inhabited those areas prior to the LGM, as it can be seen in the timing of the formation of Central-Northern European population by approximately 96,000 years. The degree of confidence in the best scenario is high, with low error rates Type I: 0.07-0.29 and Type II: 0.04-0.27 (see supplementary material S2.4). For scenario 4.6, median values for effective population sizes were 46500, 6450, 48000 and 630000 for *N1* (Spain), *N2* (Italy), *N3* (Britain) and *N4* (Continental Europe). The median values for divergence events corresponded to 616 and 4800 number of generations for *t3* and *t4*, which could be translated into approximately 12320 and 96000 years, considering Scots pine reach maturity at the age of 20 years (Carlisle & Brown 1968). The rate of admixture (*ra*) corresponded to 0.747.

2.4.2.2 Changes in population size

Nuclear markers showed the most likely scenario is a **bottleneck**, dated between 1160 and 570 generations ago. Average generation time of Scots pine have been estimated at 20-25 years, therefore the bottleneck dates would correspond to 23,000-29,000 and 11,000-14,000 years ago respectively, which is an indicative of a drastic reduction of the population size during the LGM period. Although currently those populations are currently located in Scotland, glaciology, pollen and fossil data make clear that ancestral populations must have been located either well to the south or on land now submerged on the continental shelf.

2.4.3 Species distribution modelling

All five statistical algorithms had high predictive ability and did not overfit presence data (ROC = 0.9). SDMs predicted that suitable climatic conditions for Scots pine during the LGM occurred in Iberia, Italy, the Mediterranean coast of France, the Balkans and some areas in central Europe. However, highly suitable conditions (habitat suitability > 0.7) existed mainly in the areas near the Pyrenees, Alps and the Danube plain (Fig. 2.7d,e,f,g,h,i). Suitable conditions persisted in Iberia, southern France, Italy and Danube area from the LGM until the present but between the LIG and the LGM they might have been limited to Iberia, western coast of France and the

British Isles, suggesting that these areas had potential to act as refugia across Pleistocene glaciation cycles (Fig. 2.7). Past projections showed large differences for the CCSM and MIROC circulation models, but also varied among models with different occurrence data sources (Fig. 2.7), with EUFORGEN data being the one that showed largest suitable area under past conditions (Fig. 2.7a,d,g,j) and adult NFI information the lowest (Fig. 2.7b,e,h,k).

Areas in south and mid Europe (i.e. Alps, Pyrenees, Apennines or Carpathians) appear as some of the most suitable areas for the survival of the species during the LGM. Surprisingly, however, the Danube plain, north of the widely recognised refugial areas, was indiciated as one of the largest suitable areas among all others. It is noteworthy that some areas in central Europe were also indicated as suitable (~ 50% suitability value) and even some sites on the continental western coast, near to the current coast of Ireland. Although areas in northern Europe appeared suitable regarding temperature and precipitation patterns, those areas are likely, by that time, to have had permanent ice cover sufficient to prevent tree growth

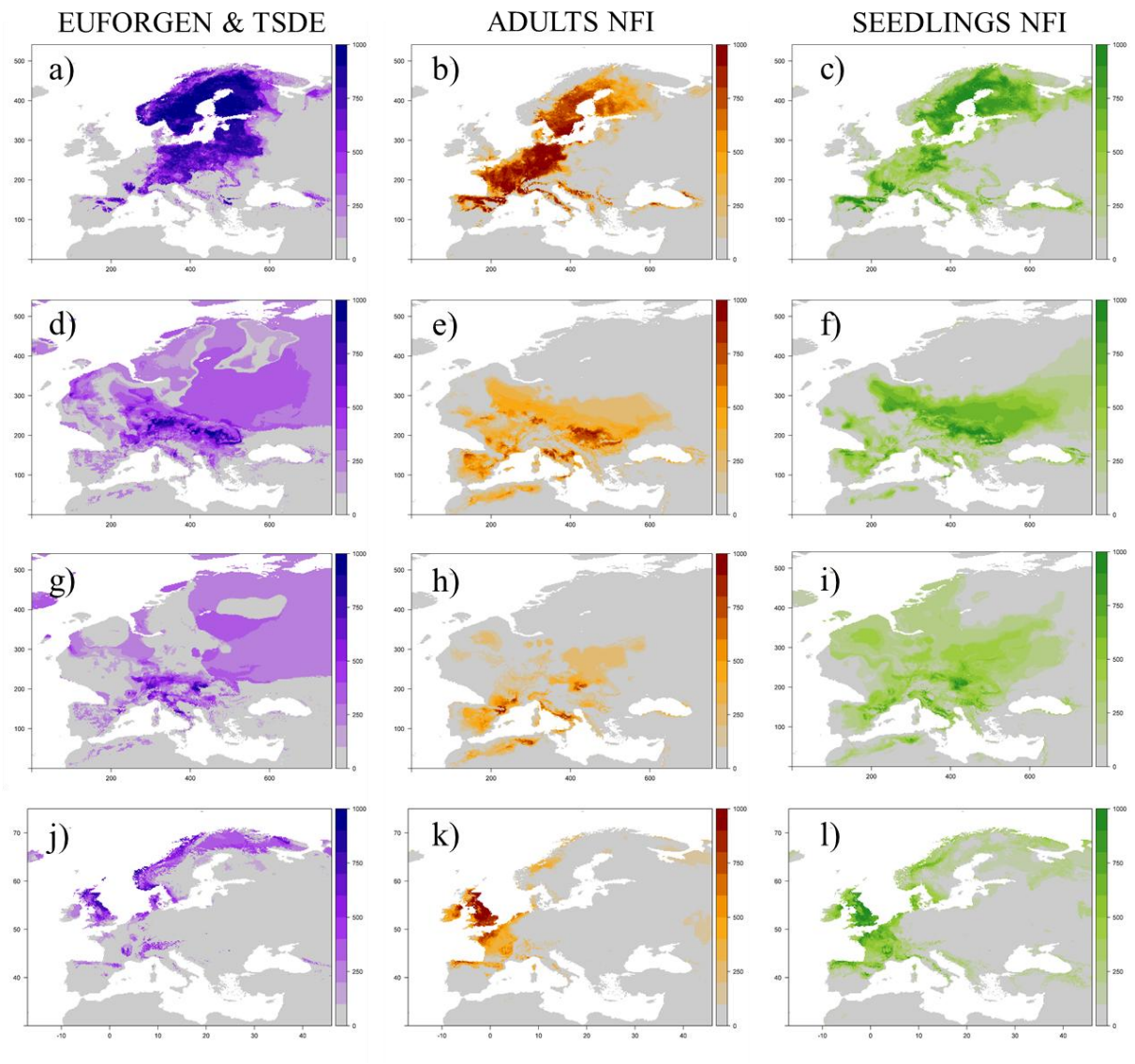

Figure 2.7 Projection of species under current climatic conditions (a,b,c), past condition in the last glacial maximum using CCSM global circulation model (i.e. LGC-CCSM; d,e,f), and MIROC global circulation model (i.e. LGC-MIROC; g,h,i), and past climatic conditions in the Last Interglacial Period (i.e. LIG (j,k,l) using different occurrence data sources: EUFORGEN and TSDE (purples), adult individuals from NFI (oranges) and regenerated individuals from NFI (greens).

2.5Discussion

Our study combined phylogeographic analysis under a Bayesian framework together with species distribution models to better understand the contribution of refugia and subsequent Holocene migrations to contemporary patterns of Scots pine distribution and genetic diversity. Our results supported (1) similar and even greater diversity in northern populations of Scots pine in comparison with southern peninsular refugia; (2) a differentiated role of peninsular glacial refugia in contributing to Holocene colonisation: Italian and Asia Minor peninsulas might have not contributed to Holocene migrations whereas Iberian and Balkan peninsulas might have, being the latter the most likely source for central-northern European populations, and (3) North-Western populations are likely derived from two genetic pools and underwent a significant bottleneck event around the time of the LGM.

2.5.1 Spatial distribution of genetic diversity at the continental scale

Our results indicated that Scots pine genetic diversity across the continent was relatively high in both nuclear and chloroplast genomes for SSR markers (nH_E=0.527 and cH_E=0.970 respectively). Such diversity estimates agree with previous studies of Scots pine through Europe (Provan et al. 1998, Robledo-Arnuncio et al. 2004, Naydenov et al. 2011, González-Díaz et al. 2017) although in some mainland European population have been reported higher (nH_E: 0.74-0.85; Scalfi et al. 2009, Nowakowska et al. 2014, García-Gil et al. 2015) and lower values (cH_E: 0.56, García-

Gil et al. 2015). We expected greater values of diversity for cpSSR compared with nSSR as a result of the nature of these markers (e.g. ploidy and inheritance mode).

We observed greater genetic diversity in central-northern European populations (i.e. Scotland, Central Europe & Fennoscandinavia) in comparison with southern peninsular populations, that was statistically significant for cpSSR (p=0.0112) but not for nSSR. This difference was in contrast to the south-north diversity model (Hewitt 1999), which suggest that generally, populations from refugial areas will harbour greater genetic diversity than areas that have been colonized postglacilly (Hewitt 2000, Tzedakis et al. 2013) and mirrored results obtained by Dering et al. (2017) and agree with (Petit et al., 2003). In Dering et al. (2017) study they attributed this departure from model south-north patterns of diversity as a consequence of the existence of contact zones from cryptic refugia in northern Europe. Accordingly, the relatively greater levels of genetic diversity, especially in cpSSR, in the Scottish and central-northern European populations from our study, might have arised from an admixture event of different genetic pools. In contrast and amongst the 23 (for European SSR) and 13 studied populations (for European SNP), Asia Minor and Italian populations respectively displayed the lowest levels of genetic diversity.

Three genetic groups were clearly distinguished through Bayesian clustering, despite that genetic differentiation was not high through the continent in terms of heterozygosity or allele richness, likely a consequence of extensive gene flow among populations. Bayesian clustering detects differences in allele frequencies and assigns

individuals to sub-populations based on analysis of likelihoods (Porras-Hurtado et al. 2013). We found marked genetic differences in the Iberian, Italian and Asia Minor peninsula, which broadly match the previously recognised refugial areas (Bennett et al. 1991, Petit et al. 2003, Naydenov et al. 2007). Notably, although the Balkan peninsula did not appear as a unique genetic cluster itself and was more related to the central-northern European group than to any of the other groups, it has also been well-recognised as a glacial refugia (Prus-Glowacki et al. 2012).

2.5.2 The role of peninsular glacial refugia in Holocene colonization

All SDM calibrated with different dataset confirmed habitat suitability for the survival of Scots pine under the LGM in areas of southern peninsulas (Fig. 2.7), although areas in Asia Minor obtained a low value of suitability. Asia Minor represents a different genetic cluster, likely carrying specific adaptations that might not be present in the other genetic lineages from our sample. Previous studies have shown that when considering aspects of local adaptation or genetic lineages in the calibration of SDM, the area suitable for the species is enlarged and usually group differentiated (Benito Garzón et al. 2011, Mayol et al. 2015, Serra-Varela et al. 2015). The lack of calibration of our models with samples from the differentiated genetic lineage of Asia Minor might have restricted the habitat suitability of the species in this area.

Phylogeographic models also supported the presence of glacial refugia in southern peninsulas, but with a different role in the subsequent colonization. Therefore, the Bayesian model suggested that refugial populations in the Italian peninsula and Asia Minor did not contribute to the Holocene colonization of Europe after the LGM, which is in agreement with previous studies. Those refugia populations faced major physical barriers, namely the Alps and Anatolian mountains, which would have limited tree dispersal and enforced stronger genetic isolation of tree populations after the LGM. This argument was also supported by the strong differentiation (see STRUCTURE cluster in Fig. 2.6 and FST values in Table 2.5) and lower values of diversity for both refugia (see nSSR and cpSSR diversity for Asia Minor in Table 2.2 and Fig. 2.5a&b, and SNP diversity for Italy in Table 2.3 and Fig. 2.5c). Previous studies have already confirmed that Italian Scots pine populations from the Apennine region are differentiated and genetically impoverished in comparison with populations from the Italian Alps populations due to their progressive isolation since the LGM (Labra et al. 2006, Scalfi et al. 2009, Belletti et al. 2012). Similarly, populations from Asia Minor, which are spatially restricted, might have been kept trapped after the LGM by the Anatolian diagonal (mountain range extending from the Anti- Taurus Mountains towards to the eastern Black Sea) (Naydenov et al. 2007, Bilgen & Kaya 2007, Dering et al. 2017), which is recognized as an important biogeographic barrier (Ansell et al. 2011). The long-term isolation of Asia Minor populations within geo- graphically separate refugia might have lead to genetic differentiation due to drift (Provan & Bennett 2008). However, some long distance

gene flow between Asia Minor and the Balkan populations might have occurred (Wójkiewicz & Wachowiak 2016).

On the other hand, we detected an Iberian contribution to Scots pine Holocene colonization. Iberian populations of Scots pine have been previously suggested to have not played role in Holocene migrations (Prus-Glowacki & Stephan 1994, Cheddadi et al. 2006, Dering et al. 2017), as the Pyrynees may have acted as an important barrier limiting gene flow of species (Razgour et al. 2013). However the possibility of some Iberian populations contributing northwards after climate ameliorated is not surprising, as although some North Eastern high elevated Iberian populations present unique mitotypes (Sinclair et al. 1999, Soranzo et al. 2000, Naydenov et al. 2007) which might indicate isolation after LGM, some other populations still retain the mitotype that dominates the remaining European forests. In fact, contribution of Iberian haplotypes to north-western populations have been already suggested in others species such as oaks (Cottrell et al. 2002, Kelleher et al. 2014). We also detected a contribution to the Balkan populations to the European Holocene colonization and in particular to the central-northern European populations. Despite being relatively isolated from the rest of the continent by mountain ranges (i.e. Balkan mountains), this result was also supported by the lowest F_{ST} estimates between Balkan and Central-Northern European populations (*nFST*= 0.00893, P>0.001; *cpFST*= -0.00047, n.s., Table 2.5) and agrees with Prus-Glowacki et al. (2012) and Cheddadi et al. (2006) who described recolonisation of

central and western European populations from Balkan populations. The argument to explain why some glacial refugia contributed to northern European colonization and others no, remain unclear. However, the specific characteristics of their surrounding mountain barriers might help to explain this difference. Pyrenees and Balkan mountains, with altitudes bounding between 2000-3000 m.a.s.l., represent lower barriers than the Alps and Anatolian mountains, almost double in altitude. Given the capacity for long distance gene flow of Scots pine (i.e. by both pollen and seed mediated gene flow) (Steven and Carlisle 1959, Robledo-Arnuncio et al. 2011), and that usually competition forces Scots pine to forest edges and onto poor quality sites (i.e. higher and colder conditions), it seems feasible that gene flow moved northwards across the lower barriers.

Furthermore, our Bayesian modelling with European SNP and SSR data suggested that some Central-Northern European populations might have been established prior to the LGM (i.e. timing of SSR data for the central European populations). Several studies have also suggested that not only southern but northern refugia of Scots pine survived during the LGM (Cheddadi et al. 2006, Naydenov et al. 2007, Buchovska et al. 2013, Donnelly et al. 2016, Dering et al. 2017 based on genetic marker, and Kullman 1998, Willis et al. 2000 based in pollen and fossil data). It seems likely that Scots pine formed a set of patchily distributed populations at mid-latitudes that were connected by gene flow extensive enough to prevent strong genetic divergence (Cheddadi et al. 2006). These patchily distributed populations

might have played an important role in recolonization (Dering et al. 2017). Given the simplicity of the scenarios tested, and as complex scenarios are limited in the ABC framework, a scenario including contributions from sparse populations at mid-latitudes could not be tested but cannot be ruled out. The existence of northern refugia was also supported by the SDM, as they showed habitat suitability not only in the southern peninsulas, but also in northern areas (i.e. northern to the Pyrenees and the Alps and the Danube plain).

2.5.3 Likely sources of the North-Western populations

Historical models with both marker types agree and strongly support the hypothesis of multiple origins for the Scottish populations. A multiple genetic pool in Scotland has been previously suggested by several authors (Forrest 1980, Kinloch et al. 1986, Ennos et al. 1997, Sinclair et al. 1998, Provan et al. 1998) but the origin of the sources were uncertain. Within the bounds of our sample and the scenarios tested, our model suggest that the Scottish populations have their origins in an admixture event between colonising fronts originating from central-northern and south western European populations (i.e. northern Iberian or southern France) previous to the subsequent British migration. In agreement, the high values of diversity for the Scottish populations (nH_E: 0.51-0.66; cH_E: 0.83-0.97), comparable to other Scottish and Eurasian populations (Provan et al. 1998, Robledo-Arnuncio et al. 2004, Naydenov et al. 2011, González-Díaz et al. 2017), are likely a result of multiple genetic pools.

These results do not exclude the possibility of the survival of some relict populations in the region of the southern British Isles or on now-submerged areas of western continental shelf, which has been previously suggested (Kinloch et al. 1986). To date, such a scenario has not been possible to test, as there are no living candidate indigenous southern British populations and very limited Scots pine pollen information from this area (Kelly et al. 2010). However, recent discoveries of native populations in Ireland might hint this finding (McGeever & Mitchell 2016) and shed more light in the origins of the most northwestern populations of Scots pine.

The latest reconstructions of the extent of British–Irish ice sheet show that Ireland was totally covered by the ice up to 21 ka BP, while southern Britain of approximately (between 52° and 53° North) was never covered by ice during the last glaciation (Hughes et al. 2016), suggesting the persistence of free-ice areas during the LGM. In agreement, SDM indicated some suitable areas for the species in southern Britain, especially under the EUFORGEN model. Nevertheless, it is important to bear in mind that existence of suitable habitat does not imply that habitat was all occupied by the species. We also found evidence of a bottleneck at the time of the LGM, with a subsequent expansion of the population, which suggest that Scots pine population size would have been reduced at this time. As expected, ecological conditions during the harsh cold and dry LGM conditions might have dramatically reduced population size and subsequently genetic variation. However, subsequent changing conditions during recolonization are also likely to have induced a series of

sequential founder effects and bottlenecks as the species moves (Provan & Bennett 2008). Therefore, the bottleneck signal detected in this study could be the result of both, the LGM itself and the subsequent colonization by a small number of individuals from southwestern and central Europe, that meet and admix.

2.5.4 Conclusions

In summary our results provide a greater resolution in the role of refuge areas into Holocene migrations of Scots pine and likely sources of colonization and suggest that historic climate changes and geographical barriers have played an important role in the spatial distribution of current genetic diversity of Scots pine. Using a combination of phylogeographic analysis together with species distribution models we were able to provide some light in the Holocene migrations. Although we agree with most previous studies in the absence of contribution from populations originated in Italy and Asia Minor, we suggest some contribution from Balkan and from the unexpected Iberian populations and likely contribution from northern refugia. In addition, we also found evidences of the multiple origins of the most northwestern populations of Scots pine. The use of multiple data sources in both phylogeographic and SDM secures the inference against bias due to data type. This has implications for studies of migration, estimation of range shifts and the role of microclimates under changing conditions. Although cryptic refugia signals are likely to have contributed to population expansion, we caution against using this evidence

as a means to classify stand origins. As new markers with greater resolution are currently developed (e.g. SNP based on a large panel of transcriptome variants), it may be possible in the near future to add more evidences of specific location from northern refugia of Scots pine populations and its role in subsequent migrations.

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2.8 Supplementary material

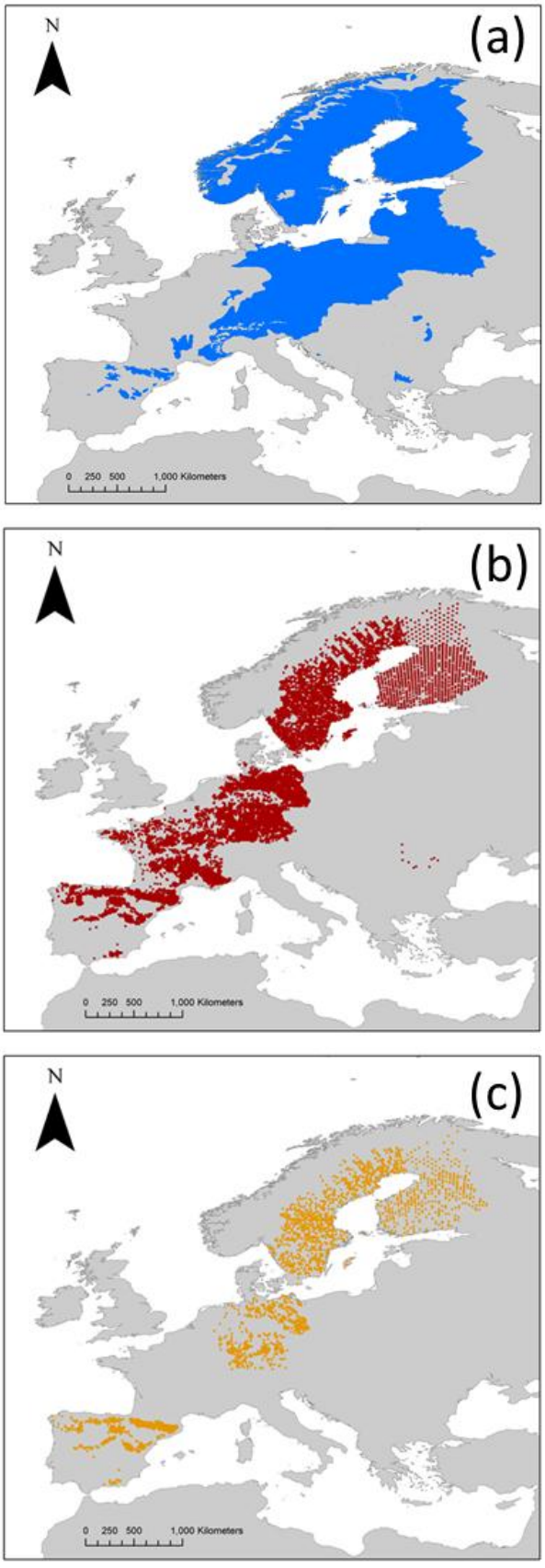
S2.1 Continental study sites for the SSR (nuclear and chloroplast microsatellite markers) European datasets

Code	Location of study site		Longitude	Latitude	Altitude (m a.s.l.)
	Country	Site name			
T1	Turkey	Şavşat- Ardahan	E 42.43	N 41.23	1700
T2		Sakaltutan Geçidi. S from Şiran	E 39.05	N 39.87	2010
T3		Tokat-Yıldızeli	E 36.52	N 39.96	1579
T4		Çatacık	E 31.11	N 39.96	1619
T5		Bayabat-Sinop	E 34.83	N 41.64	1228
U	Ukraine	Crimea. Yalta	E 34.20	N 44.55	1380
G	Greece	Ano Vrandou	E 23.65	N 41.31	1350
B	Bulgaria	Pirin. Bansko-Razlog	E 23.36	N 41.88	1076
S	Serbia	Divčibare Mts	E 19.99	N 44.10	957
SP1	Spain	-	W 2.42	N 37.01	-
S2		-	W 3.42	N 37.17	-
H1		Sierra de Gúdar. Valldelinares	W 00.61	N 40.38	1950
H2		Sierra de Neila	W 03.01	N 42.05	1400
H3		Puerto de Navafría	W 03.81	N 40.98	1800
H4		Pyrenees. Tunel de Viella	E 01.57	N 42.52	1500
A	Andorra	St. Miguel d'Engolasters	E 00.77	N 42.67	1550
F	France	Forêt Domaniale	E 02.02	N 45.66	800
IT	Italy	-	E 13.03	N 42.44	-
AU	Austria	-	E 14.55	N 47.52	-
PO	Poland	-	E 17.50	N 51.97	-
PL1		Chojnik	E 15.64	N 50.83	600
		Szczeliniec	E 16.34	N 50.48	900
PL2		Rezerwat "Pusta Wielka"	E 20.82	N 49.40	1000
		Tatry. Koryciska Wielkie	E 19.83	N 49.27	1000
		Pieniński Park Narodowy	E 20.36	N 49.42	800
		Torfowiska Tarnawa	E 22.49	N 49.10	650
PL3		Hajnówka	E 23.58	N 52.74	165
		Rezerwat Liski	E 22.82	N 51.95	150
PL4		WDN Woziwoda	E 17.91	N 53.67	120
		Rezerwat Tabórz	E 20.04	N 53.77	115
		Miłomłyn	E 19.84	N 53.76	100
FI1	Finland	-	E 29.39	N 61.76	-
FI2		-	E 23.78	N 67.33	-
F1		Joutsa	E 26.14	N 61.74	125

F2		Area near Temmes and Tyrnävä	E 25.71	N 64.69	64
F3		Area near Rovaniemi	E 26.21	N 66.57	120
F4		Area near Kielajoki	E 29.07	N 69.65	100
SW1	Sweeden	-	E 20.73	N 64.61	-
SW2		-	E 13.68	N 62.70	-
SC		Abernethy	W 3.62	N 57.21	
SC1		-	W 5.65	N 57.52	-
SC2	Scotland	-	W 2.84	N 57.06	-
SC3		-	W 3.81	N 57.17	-
SC4		-	W 4.76	N 57.34	-
SC5		-	W 4.35	N 56.67	

S2.2 Worldclim variables used as environmental predictors

Variable	Description
BIO1	Annual Mean Temperature
BIO2	Mean Diurnal Range (Mean of monthly (max temp - min temp))
BIO3	Isothermality (BIO2/BIO7) (* 100)
BIO4	Temperature Seasonality (standard deviation *100)
BIO5	Max Temperature of Warmest Month
BIO6	Min Temperature of Coldest Month
BIO7	Temperature Annual Range (BIO5-BIO6)
BIO8	Mean Temperature of Wettest Quarter
BIO9	Mean Temperature of Driest Quarter
BIO10	Mean Temperature of Warmest Quarter
BIO11	Mean Temperature of Coldest Quarter
BIO12	Annual Precipitation
BIO13	Precipitation of Wettest Month
BIO14	Precipitation of Driest Month
BIO15	Precipitation Seasonality (Coefficient of Variation)
BIO16	Precipitation of Wettest Quarter
BIO17	Precipitation of Driest Quarter
BIO18	Precipitation of Warmest Quarter
BIO19	Precipitation of Coldest Quarter



S2.3 Scots pine occurrence data from the three sources: (a) TSDE Database filtered by EURFORGEN, (b) Adult presence from national inventory dataset, and (c) regeneration presence (seedlings) from national inventory dataset.

Chapter 3

Weak isolation by distance and geographic diversity gradients persist in Scottish relict pine forest

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Under review in iForest

Photos in Chapter 3 main page taken from; left: http://www.woodlandtrust.org.uk/ (R. Whittet) and right: P. González-Díaz.

3.1 Abstract

Gene flow is one of the main factors shaping genetic diversity within and among tree populations, and occurs through pollen and seed dispersal. Recent findings of pollen-release asynchronies in distant populations of Scots pine (*Pinus sylvestris* L.) within Scotland suggest that gene dispersal among more distant populations might be less effective than previously thought. Limited gene dispersal is one of the major factors causing genetic structure for neutral markers, and pollen-release asynchrony could have driven isolation by distance (IBD) among Scottish populations. Previous studies of neutral markers found little differentiation among Scottish populations of Scots pine, however they did not consider IBD over the full Scottish range. We analysed data from 6 nuclear simple sequence repeats (SSR) and 5 chloroplast SSR loci in a total of 540 individuals of Scots pine from 18 populations across Scotland. Our aim was to assess contemporary levels and distribution of genetic variation and to test if the distribution of genetic diversity was consistent with IBD. We also analysed patterns of gene flow that could have contributed to the observed patterns of variation. Levels of genetic diversity were high, for both nuclear and chloroplast markers within populations, and there was no significant differentiation among populations. A weak signal of IBD was present. We found an increase in nuclear diversity towards the East along with greater gene flow in a West – East direction commensurate with the prevailing winds. Our findings suggest that this wind-driven gene flow is dominant over genetic drift and prevents differentiation among

the Scottish populations. It may also counteract any pollen-release asynchronies among populations.

3.2 Introduction

Genetic diversity provides the fundamental basis for the evolution of forest tree species and for their adaptation to change (FAO 2014). The importance of including genetic factors in sustainable forest management has been strongly supported in recent years (Lefèvre et al. 2013, FAO 2014, Alfaro et al. 2014, Cavers & Cottrell 2014, Fady et al. 2016b) particularly in marginal populations (Fady et al. 2016a). Accordingly, several schemes have been proposed for monitoring genetic diversity at the European (Aravanopoulos et al. 2015) and international levels (Graudal et al. 2014), including the quantification of both adaptive and neutral genetic diversity in forest trees.

Neutral genetic diversity, which has little or no effect on the phenotype, is valuable for studying the effects of historical events such as population size changes, dispersal and vicariance, and of contemporary processes affecting gene flow, such as pollen and seed dispersal (Holderegger et al. 2006). Characterisation of the level and structure of neutral genetic diversity is considered an appropriate first step to designate conservation units (Rodríguez-Quilón et al. 2016). The amount of neutral genetic diversity and how it is partitioned within and among populations typically results from the balance between gene flow and random genetic drift (Burczyk et al. 2004, Steinke et al. 2008). Gene flow can counteract genetic differentiation through genetic homogenization, whereas genetic drift (the random changes of allele frequencies over generations) is expected to lead to differentiation among

populations (Slatkin 1985). Determining levels and structure of neutral genetic diversity of tree populations should be an essential step in the design of sustainable forest management plans, as well as to better understand the processes that are likely responsible for the maintenance of such diversity.

Gene flow occurs through pollen and seed dispersal (Savolainen et al. 2007), and in wind-pollinated trees is usually more extensive by pollen than by seeds (e.g. pollen flow in pines was up to approximately 60 times greater than seed flow (Ennos 1994)). Although declining gene flow with distance is expected given that most pollen deposition, pollination events and seedling establishment occur near the parent plant (Deacon & Cavender-Bares 2015), genes and especially pollen can travel long distances, resulting in large pollen-mediated gene flow (Ouborg et al. 1999). Pollen dispersal is, therefore, expected to shape the level and structure of genetic variation within and among tree populations at local and regional scales (Burczyk et al. 2004). For example, Scots pine pollen from southern and central Finland contributes to populations located hundreds of km further north (Lindgren et al. 1995, Varis et al. 2009).

Multiple ecological factors can restrict pollen-mediated gene flow among wind-pollinated tree populations, and hence lead to temporal or spatial increases in genetic drift. Physical barriers (e.g. mountains) (Naydenov et al. 2011) and fragmentation (Provan et al. 2007, Cuartas-Hernández et al. 2010) may restrict pollen flow. However, in some cases, increases of pollen flow counteract diversity loss

resulting from fragmentation (White et al. 2002, Petit & Hampe 2006, Wang et al. 2012, Davies et al. 2013). Other significant barriers to pollen flow, that have received less attention, can be due to asynchrony in reproductive phenology (e.g. timing of pollen or flower production) (Aitken et al. 2008, Whittet et al. 2017), which may limit the randomness of mating (Gutierrez & Sprague 1959). Indeed, there is evidence that plants mate with phenologically similar individuals more frequently than random (Gutierrez & Sprague 1959, Ennos & Dodson 1987). Consequently, differences in timing of reproductive phenology in nearby tree populations are expected to favour mating between physically closer individuals by mating incompatibility among tree populations, thereby reinforcing isolation by distance (IBD). In addition, wind patterns might also influence the levels and direction of gene flow, such as the significant contribution to directional pollen flow in Scots pine populations from Scandinavia (Lindgren et al. 1995).

The fragmented natural Scots pine (*Pinus sylvestris* L.) forests of Scotland represent the westernmost extreme of the species' native range, separated by at least 500 km from natural stands in mainland Europe (Ennos et al. 1997b). Despite their geographical marginality, previous studies on the Scottish Scots pine populations found high levels of neutral genetic variation but little differentiation among populations (Provan et al. 1998, Sinclair et al. 1998, Wachowiak et al. 2011) (Tab. 3.1). The westernmost populations have previously been found to be somewhat distinct

from the others (Forrest 1980, Kinloch et al. 1986, Sinclair et al. 1998) (Table 3.1), on the basis of allele frequency differences.

A recent study found mismatches in timing of pollen release between a Western and an Eastern population from Scotland. Pollen was released first in the west, between 9.8 to 15.8 days earlier than in the east (Whittet et al. 2017). Consistent asynchrony in pollen production among populations will limit gene flow; where the extent of asynchrony reflects geographic distance, a pattern of IBD will be established. Although several studies have addressed genetic variation in Scots pine from Scotland (Table 3.1), these studies have not investigated patterns of IBD in the full Scottish range. Forrest (1980) found a trend of gradually increasing genetic similarity from the southwest of the Scottish range toward the northeast. Other studies included estimates of F_{ST} (Kinloch et al. 1986, Sinclair et al. 1998, Wachowiak et al. 2011, Wachowiak et al. 2013), which indicate the extent of genetic differentiation (genetic structure) among populations, regardless of the distance. Measures of IBD indicate the relationships between geographic and genetic distance, and can help us to identify where restricted gene dispersal may relate to geographic factors.

Based on the previous evidence, we hypothesised that, despite extensive pollen dispersal by wind, the presence of an East-West asynchrony in pollen production might result in IBD due to a higher probability that more synchronous populations will mate with each other. Alternatively, the absence of identifiable IBD would suggest that despite the presence of pollen asynchrony, effective gene flow at the

regional scale, might have prevented differentiation. To test this hypothesis we characterised genetic diversity along the full East-West gradient of Scots pine within Scotland with two sets of neutral molecular markers, nuclear and chloroplast microsatellites (SSR). The use of both markers can allow greater understanding of the factors driving differentiation than based on either alone (Zinck & Rajora, 2016, Sjölund et al. 2017); chloroplast DNA is paternally inherited in conifers via dispersing pollen, and nuclear DNA is bi-parentally inherited via both pollen and seed dispersal. In addition, the coverage of the full native range of Scots pine in Scotland, allows much greater resolution of geographical structuring of gene flow, including IBD, since most other SSR-based studies of this species incorporated relatively few Scottish populations (Provan et al. 1998, González-Díaz et al. 2017) or did not focus on these aspects of the species' biology (e.g. Salmela et al. 2011). Specifically, we sought to answer the following questions, (1) what are the contemporary levels and structure of genetic variation of the Scots pine populations across Scotland, (2) is there any evidence of IBD and (3) what are the gene flow patterns among the studied populations?

Table 3.1 Studies assessing neutral genetic variation of Scots pine in Scotland using variable molecular markers: RFLP, Restriction Fragment Length Polymorphism; SSR, Simple Sequence Repeat or microsatellite; SNP, Single Nucleotide Polymorphism, indicating No. pop., number of studied populations; No. ind., number of genotyped individuals.

Marker	Location	No. pop.	No. ind.	No. markers	Population differenciation Gst*/Fst**/ AMOVA#/	Diversity	Reference	Main conclusions
Monoterpene	Scotland	41	6705	11	Similarity matrix: 0-24 over 30	-	Forrest et al., 1980	Variation between sites allowed divide the natural range into several areas of biochemical similarity, the most distinct being a north-western group of sites with Shieldaig as its most distinctive site. A trend of gradually decreased of similarity from the north east from Scotland in a south westerly direction.
Monoterpene	Europe	6	953	11	Similarity matrix: 3-24 over 30	-	Forrest 1982	Northern European populations were similar to each other but the three from middle and southern Europe showed large differences from them and from each other. Western region from Scotland showed similarity to middle Europe and south western populations to northern Europe.
	Scotland (from Forrest et al., 1980)	41	953	11	Similarity matrix: 0-24 over 30	-		
Monoterpene	Scotland	40	5765	6	0.045*	0.272 - 0.378	Kinloch et al., 1986	High genetic diversity. Several populations in the Western region from Scotland were distinct from all others and each other. Scottish Scots pine forest originated from more than one refugium after the last glaciation.
Izozymes		14	2177	9	0.028*	0.291 - 0.311		
RFLP (mtDNA)	Scotland	20	466	Coxl mitochondrial gene	0.37**	-	Sinclair et al., 1998	Two mitotypes were present: mitotype a is present at all sites, but that mitotype b is confined to three western populations
SSR (cpDNA)	Scotland	7	330	17	0.032#	0.950 - 0.987	Provan et al. 1998	Higher levels of diversity for the Scottish populations than those for European population. A mutation in one loci occurred in the Western region of Scotland.
	Europe	8	185	17	Scot vs Eur 0.0148#	0.908 - 0.976		
SNP (nDNA)	Scotland	21	42	16	-0.017 - 0.023	0.754 - 0.819 (0.831 at 8 loci)	Wachowiak et al., 2011	High genetic diversity
	Europe	7	40	10	0.071 - 0.079	(0.795 at 8 loci)		
SSR (nDNA)	Scotland	21	1680	3	-	-	Salmela et al., 2011	High levels of outcrossing in Scottish populations
SNP (nDNA)	Scotland	12	120		0.009**	0.67	Wachowiak et al., 2013	High levels of nucleotide diversity within populations
SNP (mtDNA)	Scotland					0.81		
SSR (nDNA)	Scotland	2	647	12	0.004**	0.56-0.58	González-Díaz et al. 2017	High levels of genetic diversity and presence of moderate fine-scale spatial genetic structure

3.3 Material and methods

3.3.1 Study species

Scots pine (*Pinus sylvestris* L.) in Scotland is a foundation species of the remnant Caledonian pine forests. It is typically a pioneer species that readily regenerates after major natural or human disturbances, if competition and grazing pressure are low (Mátyás et al. 2004). It grows well on most soils, nevertheless, due to low tolerance of shade and competition, it is often restricted to poor soils (Steven & Carlisle 1959). Based on fossil data, this species reached its maximum extent in Scotland around 8000 years ago, covering approximately 1.5 million ha (Bennett 1984, Birks 1989, Froyd 2006). Nowadays, 84 fragments of the ancient native pine forest remain in this area, scattered over a total area of 17,882 ha (Mason et al. 2004).

3.3.2 Study sites and microsatellite analysis

Eighteen populations were selected to cover the full native range of Scots pine within Scotland (Table3.2, Fig. 3.1). We sampled a total of 30 randomly selected trees within each population. Sampled trees were old adults, thereby avoiding potential effects of gene flow from more recent Scots pine plantations that might otherwise obscure patterns of genetic diversity and divergence in the native stands.

Figure 3.1 Study populations for nSSR and cSSR analyses.

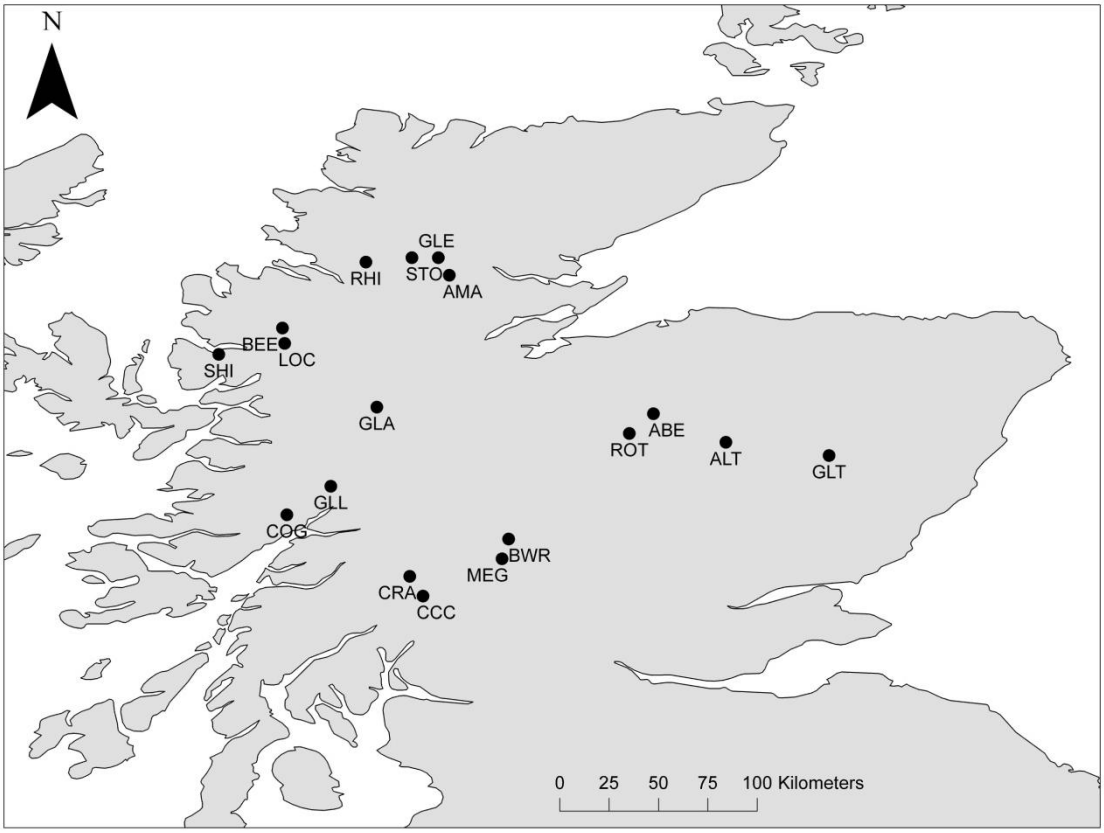


Table 3.2 Details of study sites. Population area was obtained from Mason et al. (2004).

Population name	Population code	Seed zone	Population pine area	Latitude	Longitude	Altitude
Cona Glen	COG	SW	189	56.78	5.33	148
Glen Loy	GLL	SW	74	56.91	5.13	170
Crannach	CRA	SW	70	56.5	4.77	296
Coille Coire Chuilc	CCC	SC	67	56.41	4.71	257
Meggernie	MEG	SC	277	56.58	4.35	306
Black Wood of Rannoch	BWR	SC	1011	56.67	4.32	275
Abernethy	ABE	EC	2452	57.24	3.66	341
Rothiemurchus	ROT	EC	1744	57.15	3.77	318
Allt Cul	ALT	NE	13	57.11	3.33	476
Glen Tanar	GLT	NE	1564	57.05	2.86	334
Glen Affric	GLA	NC	1532	57.27	4.92	256
Amat	AMA	NC	181	57.87	4.59	137
Loch Clair	LOC	NW	126	57.56	5.34	132
Shieldaig	SHI	NW	103	57.51	5.64	81
Beinn Eighe	BEE	NW	182	57.63	5.35	63
Glen Einig	GLE	N	27	57.95	4.76	55
Strath Oykell	STO	N	14	57.95	4.64	103
Rhidorroch	RHI	N	103	57.93	4.97	182

Total genomic DNA was extracted from 50 mg silica gel-dried needles using a QIAGEN DNeasy plant extraction kit (QIAGEN Ltd. Crawley, UK) following the manufacturer's protocol. All individuals from the eighteen Scottish populations were genotyped at six nuclear (nSSR) and five chloroplast (cSSR) microsatellite markers. We used six nSSR: PSAJ223770/SPAC11.14, PSAJ223766/SPAC11.8 (Soranzo et al. 1998), Ptx2146 (Aukland et al. 2002), SsrPt_ctg4698, SsrPt_ctg9249 (Chagné et al. 2004) and LOP3 (Liewlaksaneeyanawin et al. 2004); and five cSSR: PCP26106, PCP30277, PCP36567, PCP45071, PCP87314 (Provan et al. 1998). Reactions were carried out in a final volume of 10 μl with 1 μM fluorescently-labelled forward primer, 1 μM reverse primer, 200 μM each dNTPs, 0.5 units Taq polymerase (Roche Applied Science), 1X PCR buffer (supplied with Taq) and 25 ng of template DNA. Annealing temperature was 56°C. Polymerase chain reactions (PCR) were performed in a Gene Amp PCR System 9700 thermo cycler (Applied Biosystems, Bleiswijk, Netherlands), with the following programme: 1 cycle at 95°C for 4 min followed by 35 cycles at 95°C for 45 s, 56°C for 45 s, 72°C for 45 s, and a final step at 72°C for 5 min. Fragment analysis was performed by Genome Technology at James Hutton Institute, Dundee, UK, using a 3730 DNA Sequencer (Applied Biosystems) with reference to a ROX 500 size standard. Fragments were scored using GeneMarker V.2.6.0. (SoftGenetics, State College, PA, USA). Null allele frequencies at nuclear loci for each locus and each population were checked by using the software Micro-Checker (Van Oosterhout et al. 2004). PSAJ223770/SPAC11.14 showed evidence of null alleles, however the frequency was below 0.2, which is the threshold over which

null alleles can result in a significant underestimate of expected heterozygosity H_E (Chapuis & Estoup, 2007, Belletti et al. 2012), therefore it was kept for further analysis.

3.3.3 Data analysis

3.3.3.1 Genetic diversity

Genetic diversity estimators within populations were estimated using FSTAT 2.9.3.2 (Goudet 1995) and Arlequin v3.5 (Excoffier & Lischer 2010). For nSSR, we estimated mean number of alleles per locus (*nA*), rarefied allelic richness (nA_R), number of private alleles (*nAp*), observed heterozygosity (nH_O), expected heterozygosity (nH_E) and inbreeding coefficient (nF_{IS}). Rarefaction of allelic richness controls for differences in sample size (El Mousadik & Petit 1996) allowing comparison among sites and studies. For cSSR, we estimated mean number of alleles (*cA*). For the remaining cSSR estimators, alleles were combined to compose a unique chloroplast haplotype for each individual. Individuals with missing data were discarded from the inference of multilocus haplotypes. We estimated number of haplotypes (cH_N), number of private haplotypes (cH_P) and gene diversity (cH_E), the latter based on haplotype frequencies (Nei, 1987).

3.3.3.2 Population differentiation and Bayesian clustering

To estimate population differentiation, we calculated nF_{ST} among populations using nSSR in Arlequin v3.5 (Excoffier & Lischer 2010), and the differentiation index *D* (Jost 2008) implemented in the R package DEMEtics (Gerlach et al. 2010). In both cases, significance values were determined for a 5% nominal level after Bonferonni correction. Multilocus haplotypic and genetic estimators for both nSSR and cSSR (nH_E, nA_R, cH_E and cH_N) were mapped using ARCMAP 10 (ESRI, Redlands, CA, USA), using inverse distance weight methods available on the spatial analyst interpolation tool. To map nuclear genetic differentiation, for each site we calculated the percentage of total sites that it was significantly differentiated from (e.g. percentage of differentiated sites, *nDS* (%)), based on nF_{ST} values (Weir & Cockerham 1984). To quantify the distribution of variation of nuclear genetic diversity and chloroplast haplotypes, we tested both marker sets in an hierarchical analysis of molecular variance (AMOVA) from the level of individual, population and cluster of populations (see directional relative migration rates section), performed in Arlequin 3.5 (Excoffier & Lischer 2010).

We performed individual-based Bayesian assignment methods using data from nuclear loci in STRUCTURE 2.3.4. (Pritchard et al. 2000). We used a model assuming correlated allele frequencies (Falush et al. 2003) and admixed ancestry. We included the site location *a priori* (LOCPRIOR option) to improve the detection of weak population structure (Hubisz et al. 2009). *K* was set from 1 to 20, with 10 runs

performed for each value of *K*. Runs consisted of 500,000 Markov Chain Monte Carlo (MCMC) iterations with a burn-in period of 100,000. We used STRUCTURE HARVESTER (Earl & VonHoldt 2011), an application that uses the Evanno method (Evanno et al. 2005) for assessing and visualizing likelihood values across multiple values of *K* and detecting the number of genetic groups that best fit the data.

3.3.3.3 Isolation by distance and directional relative migration rates

For testing isolation by distance we used nuclear markers in SPAGeDi 1.4 (Hardy & Vekemans 2002) with significance determined by permuting site locations among populations 10,000 times. Following Rousset (1997), we used the F_{ST} /(1- F_{ST}) ratio as a measure of genetic distance as it is expected to vary linearly with the natural log of the geographical distance.

To reduce the number of sites to an analytically tractable set for estimating migration patterns, we grouped sites with their nearest neighbours to give seven site clusters, corresponding to the biochemical zones described by Forrest (1980) and used as a seed zones (Table 2). Directional relative migration rates between site clusters were estimated using nuclear markers and the function DIVMIGRATE from the R-package DIVERSITY (Keenan et al. 2013) using JOST'S D (Jost 2008) as a measure of genetic differentiation. To test whether relative migration is significantly higher in one direction than the other (e.g. asymmetric), 95% confidence intervals were calculated from 1,000 bootstrap iterations.

3.4 Results

3.4.1 Genetic diversity

Among the six nuclear loci analysed, the number of alleles per locus (*nA*) ranged from 2 to 14, with a multilocus average of 11.5 ± 7.34 for all populations combined. In total we obtained 9 private alleles. For rarefied allelic richness (nA_R), multilocus estimates ranged from values of 5.42 to 7.55, based on a minimum number of 28 diploid individuals, with a multilocus average of 5.84 for all populations combined (Table 3.3 and Fig. 3.2b). The multilocus expected (nH_E) and observed heterozygosity (nH_O) was 0.62 ± 0.03 and 0.54 ± 0.04 respectively for all populations combined (Table 3.3, and Fig. 3.2a for nH_E). A general trend of lower nuclear diversity (nH_E) in the western sites and relatively higher diversity in the eastern sites was observed (Fig. 3.2a). This trend was positively correlated with longitude ($F=6.703$, $R^2=0.25$, $p<0.05$), but not for nA_R. A general significant homozygote excess was found (overall $F_{IS}=0.121$, $p < 0.05$) (Table 3.3).

Among the five chloroplast loci analysed, the number of alleles per locus (c*A*) ranged from 2 to 6, with a multilocus average of 3.08 for all populations combined. Gene diversity (cH_E) ranged between 0.83 and 0.96 (Table 3.3 and Fig. 3.2c). The number of haplotypes (cH_N) within populations ranged from 13 to 19 (Table 3.3 and Fig. 3.2d), with a total of 64 haplotypes recorded, of which 42% (27 haplotypes) were private (unique to a particular population) (Table 3.3). Although most western sites showed high levels of chloroplast gene diversity (cH_E), some of the eastern sites

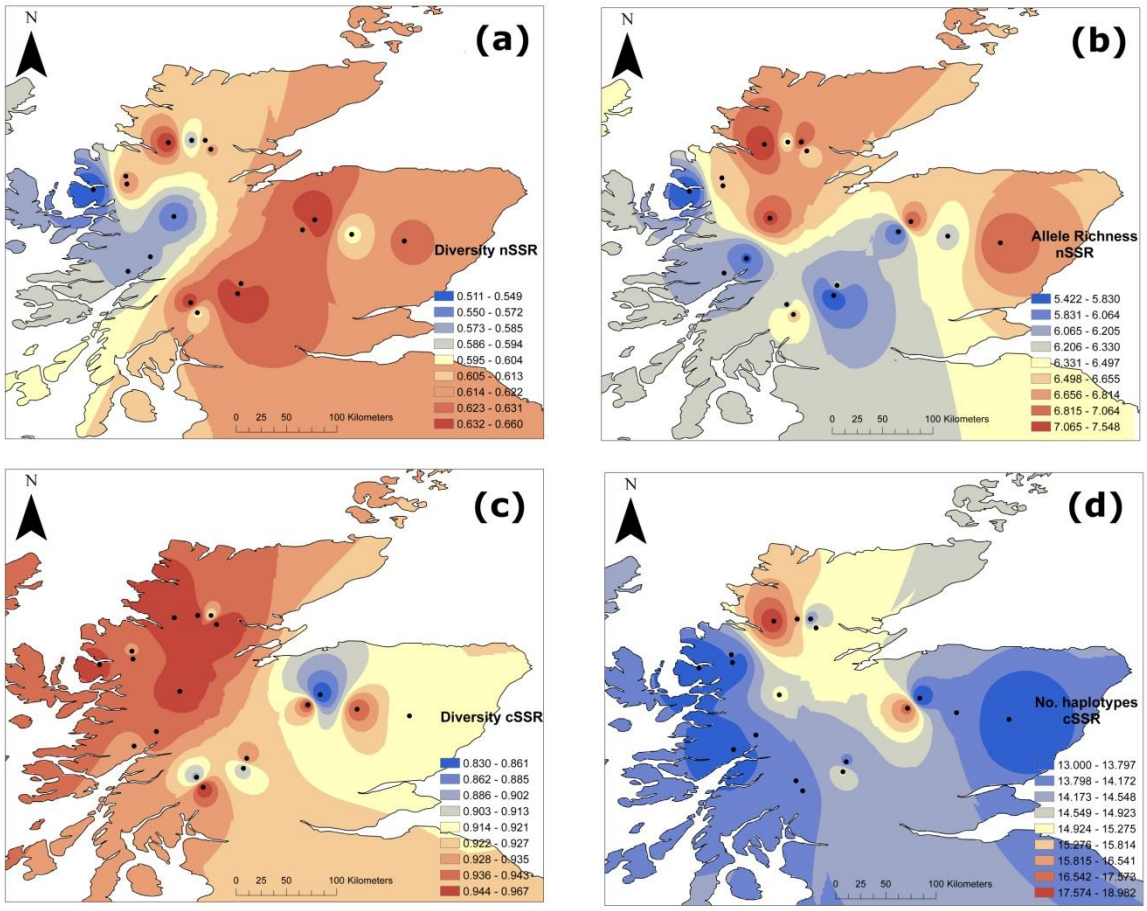
showed also high levels of diversity and the spatial trend was not as clear as for nSSR (Fig. 3.2c). No significant correlation with longitude was found for chloroplast diversity.

Table 3.3 Genetic diversity estimators for nuclear (nSSR) and chloroplast (cSSR) markers.

Population	nSSR							cSSR				
	nN	*nA*	nA_R	*nAp*	nH_O	nH_E	nF_{IS}	*cN*	*cA*	cH_N	cH_P	cH_E
COG	30	6.1670 (3.3120)	6.0992 (2.9791)	1	0.4944 (0.2352)	0.5779 (0.2402)	0.1460***	28	3.2 (1.095)	13	4	0.9339 (0.0227)
GLL	30	5.8330 (2.9270)	5.7860 (2.6523)	0	0.4944 (0.1500)	0.5801 (0.2743)	0.1500***	25	2.8 (0.837)	14	1	0.9400 (0.0280)
CRA	30	6.3330 (3.1410)	6.2860 (2.8557)	1	0.5151 (0.1960)	0.6389 (0.1990)	0.1960***	25	3.0 (1.225)	14	1	0.8967 (0.0431)
CCC	30	6.6670 (4.2270)	6.5777 (3.7589)	0	0.5722 (0.0460)	0.5995 (0.2201)	0.0460	23	2.8 (1.304)	14	1	0.9565 (0.0220)
MEG	30	5.5000 (2.8110)	5.4753 (2.5497)	0	0.5556 (0.1570)	0.6573 (0.1552)	0.1570***	28	3.4 (1.673)	15	3	0.9048 (0.0420)
BWR	30	6.3330 (2.9440)	6.2638 (2.6361)	0	0.5860 (0.0560)	0.6200 (0.1885)	0.0560	23	3.2 (1.304)	14	3	0.9368 (0.0331)
ABE	30	7.1670 (3.7640)	7.0532 (3.3582)	1	0.6111 (0.0590)	0.6490 (0.2224)	0.0590	27	2.8 (1.304)	13	0	0.8291 (0.0684)
ROT	30	5.8330 (2.3170)	5.7767 (2.0727)	1	0.5935 (0.0500)	0.6240 (0.2063)	0.0500	27	3.4 (1.140)	17	3	0.9487 (0.0257)
ALT	30	6.3330 (3.0770)	6.2532 (2.7636)	1	0.5937 (0.0120)	0.6006 (0.2542)	0.0120	25	2.8 (0.837)	14	0	0.9433 (0.0240)
GLT	30	7.1670 (2.9270)	7.0615 (2.5983)	0	0.5556 (0.1100)	0.6228 (0.2215)	0.1100*	25	3.4 (1.140)	13	1	0.9167 (0.0349)
GLA	30	7.3330 (4.2270)	7.1470 (3.7213)	0	0.4925 (0.1170)	0.5565 (0.2660)	0.1170**	22	3.4 (0.241)	15	2	0.9524 (0.0291)
AMA	30	6.5000 (3.6740)	6.4505 (3.3049)	0	0.5444 (0.1160)	0.6149 (0.2604)	0.1160**	21	3.2 (0.837)	15	1	0.9667 (0.0236)
LOC	30	6.6670 (3.4450)	6.5968 (3.0930)	0	0.5803 (0.0690)	0.6226 (0.2130)	0.0690	27	3.0 (1.000)	13	0	0.9373 (0.0220)
SHI	30	5.5000 (3.2710)	5.4198 (2.9151)	0	0.4944 (0.0330)	0.5110 (0.2737)	0.0330	29	3.0 (0.707)	13	2	0.9458 (0.0173)
BEE	30	6.6670 (3.7240)	6.6082 (3.3824)	1	0.5500 (0.1040)	0.6128 (0.2978)	0.1040*	29	3.2 (1.304)	14	0	0.9335 (0.0228)
GLE	30	6.5000 (2.8810)	6.3923 (2.5723)	2	0.4571 (0.2140)	0.5794 (0.2517)	0.2140***	28	2.8 (0.837)	16	2	0.9497 (0.0214)
STO	30	7.3330 (3.5020)	7.1848 (3.1003)	0	0.5222 (0.1450)	0.6095 (0.2079)	0.1450***	27	2.8 (1.304)	14	1	0.9145 (0.0334)
RHI	30	7.6670 (3.5020)	7.5522 (3.1469)	1	0.5714 (0.1130)	0.6430 (0.2847)	0.1130*	30	3.2 (1.304)	19	2	0.9586 (0.0209)
All populations	540	11.5000 (7.3420)	5.8400 (3.1362)	9	0.5434 (0.2068)	0.6179 (0.2327)	0.121***	469	4.6 (1.140)	64	27	-
Overall mean	30	6.5278 (3.3152)	6.4435 (2.9700)	0.5	0.5456 (0.1101)	0.6067 (0.2334)	0.1052	26.05	3.1 (1.077)	14.44	1.5	0.9315 (0.0298)

nN, no. of samples genotyped with nSSR; *nA,* no. of alleles; nA_R rarefied allelic richness for 28 diploid individuals; *nAp* no. of private alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, inbreeding coefficient; c*N,* no. of samples genotyped with cSSR; *cA*, no. of alleles; cH_N, no. of haplotypes; cH_P, no. of private haplotypes and cH_E, gene diversity corrected for sample size (Nei, 1978). Significant *P*-values are indicated as **P* < 0.05; ***P* < 0.01; ****P* < 0.001. *P*-values for F_{IS} are obtained after 1,000 permutations of gene copies within individuals of each stand. Standard errors are reported in brackets.

Figure 3.2 - Genetic diversity parameters for nSSR (above) and cSSR (below). (a) Genetic diversity for nSSR (nH_E); (b) Rarefied allele richness for nSSR (nA_R), (c) Gene diversity corrected for sample size for cSSR (cH_E); (d) no. of haplotypes for cSSR (cH_N).



3.4.2 Population differentiation and Bayesian clustering

When testing differences among populations, multilocus nF_{ST} values ranged from -0.005 (ROT and GLT) to 0.065 (SHI and MEG), being significant ($p<0.05$) in most cases (see supplementary material S3.1). Thus, for instance, of the most differentiated populations, SHI was significantly different from all populations and BWR from all but GLA (see supplementary material S3.1 and S3.2). RHI, BEE, CCC, MEG and ALT significantly differed from all but two or three populations (see supplementary material S3.1 and S3.2). Jost´s D values were in agreement but

greater than F_{ST} values and ranged between -0.012 (ROT and GLT) to 0.127 (SHI and MEG). Interestingly, the populations with the greatest asynchrony in pollen release (Whittet et al. 2017), BEE and ALT, had some of the largest F_{ST} values (FST=0.05, p<0.05) (see supplementary material S3.1). The AMOVA results showed that although most of the variation was found within populations, among-population variability is greater within groups than between them for both sets of molecular markers (Table 3.4).

Table 3.4. Hierarchical analysis of molecular variance (AMOVA) for nuclear (nSSR) and chloroplast (cSSR) markers at the individual, population and cluster of populations. The degrees of freedom (*df*), percentage of variation explained by each level (Variation (%)), and the relevant *P*-values are indicated.

Source of variation	nSSR			cSSR		
	d.f.	Variation (%)	*p*-value	*d.f.*	Variation (%)	*p*-value
Among cluster of populations	6	0.17	0.25	6	0.25	0.11
Among populations	11	1.79	<0.001	11	1.89	<0.001
Within populations	1062	98.04	<0.001	453	97.86	<0.001

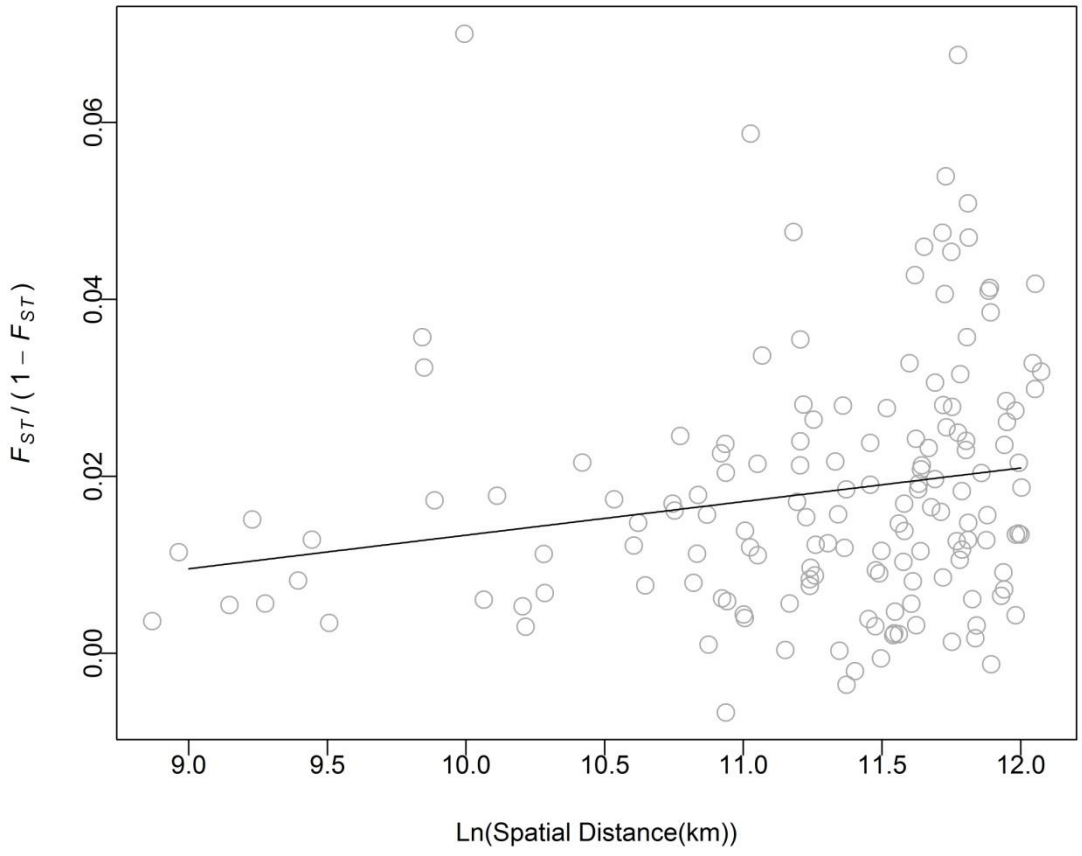
Structure identified *K*=2, as the more likely number of clusters (red and blue clusters hereafter), however, these clusters were not related to the East-West location of the individuals (see supplementary material S3.3). Most sites contained highly admixed individuals; however, the site SHI had more than 75% of individuals in the less common blue cluster. In addition, four sites had between 60 and 75% of individuals in the blue cluster STO, COG, BWR, ROT, ALT, followed by

LOC, GLA and GLT with more than 50%. All the other sites had a majority of the red cluster.

3.4.3 Isolation by distance and asymmetric migration

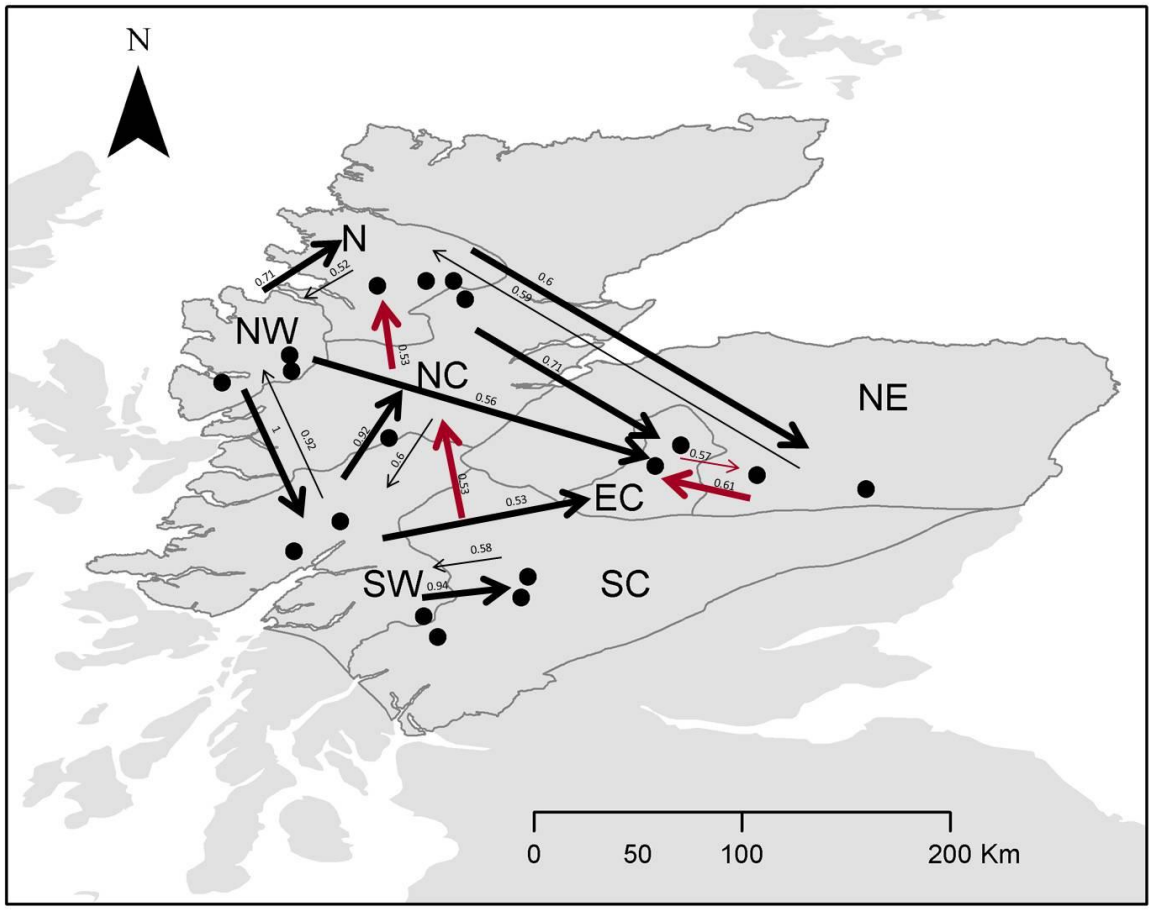
Although weak, IBD was significant in the Scottish populations (slope = 0.0038, R^2 = 0.035, $P < 0.05$) (Fig. 3.3), indicating that when geographic distance increases, populations became more differentiated. Expressing geographic and/or genetic distance on a logarithmic scale did not improve the model fit (data not shown).

Figure 3.3 - Isolation by distance. Black line represent the slope of the correlation of the natural log of the linear spatial distance (Ln(Spatial Distance (km)) against $F_{ST}/(1-F_{ST})$ after 10,000 permutations of sites among locations.



The relative migration network (see supplementary material S3.4a) shows all relative migration rates between site clusters of Scottish populations of Scots pine. Those rates indicate that gene flow was, in most cases, greater in the West-East direction than in the East-West direction, although it was not significantly asymmetric. In Fig. 3.4 and supplementary material S3.4b, directional relative migration rates below 0.50 were filtered out to emphasize the major gene flow networks.

Figure 3.4 - Relative migration networks for nSSR with populations sorted in seed groups (see Table 3.2 for population details) using a threshold of 0.5. Arrows in black denote greater gene flow pattern in the W-E direction, whereas arrows in red denote greater gene flow in the E-W or S-N direction. Thicker arrows mean stronger gene flow in such direction.



3.5 Discussion

Our study presents a detailed genetic survey of a subset of the remaining natural populations of Scots pine from Scotland using both bi-parentally inherited nuclear SSR and paternally inherited chloroplast SSR. Three main results were obtained: i) high levels of genetic variation and low population differentiation, ii) a weak pattern of isolation by distance, and iii) an increase of nuclear diversity towards the East. While we detected some discrepancies (e.g. SHI), our findings suggest that the effects of gene flow dominate those of genetic drift and prevent differentiation in the Scottish populations. Our results suggest greater gene flow in the West-East direction, likely influenced by prevailing wind patterns.

3.5.1 High levels of genetic variation and low population differentiation

In agreement with other molecular markers (Forrest 1980, Kinloch et al. 1986, Sinclair et al. 1998, Provan et al. 1998, Wachowiak et al. 2011), our results indicated relatively high levels of genetic variation across the Scottish native populations of Scots pine for both nuclear and chloroplast microsatellite markers. This is counter to expectations given their marginal distribution in global terms and the severe reduction in the extent of forest, and its fragmentation in recent centuries. Values of multilocus nuclear SSR diversity (nH_E: 0.51-0.66) were comparable to those found in other Scottish and Eurasian populations (nH_E: 0.50-0.69) (Naydenov et al. 2011,

Bernhardsson et al. 2016, Toth et al. 2017, González-Díaz et al. 2017), although lower than in some mainland European populations (*nH*$_E$: 0.74-0.85) (Scalfi et al. 2009, Nowakowska et al. 2014, García-Gil et al. 2015). In addition, the often high levels of chloroplast SSR diversity (*cH*$_E$: 0.83- 0.97) were similar to those reported for other Scottish, Iberian and Italian populations (*cH*$_E$: 0.92-0.99) (Provan et al. 1998, Robledo-Arnuncio et al. 2004a, Scalfi et al. 2009) although somewhat higher than those reported from Finland (*cH*$_E$: 0.56) (García-Gil et al. 2015). Some authors have hypothesised multiple origins for the Scottish Scots pine population (e.g. through contribution of cryptic glacial refugia) (Forrest 1980, Kinloch et al. 1986, Ennos et al. 1997b, Sinclair et al. 1998, Provan et al. 1998). If true, this might help to explain the high levels of diversity we observed, reflecting a process of admixture following secondary contact.

We detected low levels of population differentiation, which represented less than 2% of the genetic variation among populations for both nuclear and chloroplast markers (Table 3.4). Outcrossing, long-lived trees with wind-mediated gene dispersal mechanisms usually harbour more diversity within populations than among them (Hamrick et al. 1992). High gene flow among populations counteracts differentiation due to drift and maintains levels of diversity (Slatkin 1985). Levels of differentiation among populations were very similar for both marker types (1.89% vs. 1.79% for chloroplast and nuclear markers respectively). Although chloroplast loci are expected to show greater differentiation than nuclear loci due to

uniparental inheritance, smaller effective population size and higher susceptibility to genetic drift (Lendvay et al. 2014). However, the extensive pollen flow characteristic of conifers is likely to smooth such differences (Ribeiro et al. 2002).

Despite evidence of extensive gene flow and consequently weak overall genetic structure, some significant differentiation among populations was apparent. In particular, the most western population SHI was most differentiated from other populations (greatest F_{ST}). The distinctiveness of this population has been reported by other authors (Forrest 1980, Kinloch et al. 1986, Sinclair et al. 1998), although the basis for the difference remains uncertain. Some authors have observed unique, low frequency organelle haplotypes in western populations (Kinloch et al. 1986), suggesting contributions from a western refugium and possibly accounting for its distinctiveness. However, other local factors could explain the difference and resolution of the question awaits markers with sufficient power to conclusively characterise the postglacial colonisation patterns within Scotland.

3.5.2 Weak isolation by distance

Our results showed a weak but significant pattern of IBD across Scotland. IBD occurs as a consequence of limited gene dispersal such that populations close to each other tend to be more genetically similar than populations farther apart (Wright 1943). Several factors are likely to limit seed and pollen flow among the Scottish populations of Scots pine. Firstly, the recently characterised asynchrony in

pollen phenology between eastern and western populations within Scotland (Whittet et al. 2017) might limit gene transfer among populations even if pollen dispersal is physically possible across the distances concerned. Secondly, the drastic reduction in the effective population sizes and increased population isolation resulting from widespread deforestation in Scotland (Mason et al. 2004) may simply have reduced pollen availability to individual populations. Thirdly, the prevailing wind direction at the time of pollen release in Scotland is from the southwest (Dore et al. 2006). The resulting directional bias in pollen flow may enforce pollen limitation in populations to the west. Given the weak magnitude of IBD and since the described factors might probably contribute simultaneously to the observed pattern, we cannot unambiguously ascribe IBD patterns to a particular mechanism. On the other hand, if the weak magnitude of the IBD signal is driven by relatively recent pollen-release mismatches, then IBD might be expected to be exacerbated in future generations. Gene flow is nevertheless clearly effective in minimising isolation, and IBD is limited.

Some of the Scottish populations showed significant levels of inbreeding (F_{IS}= 0.11-0.21), indicating an excess of homozygotes. Other studies obtaining similar inbreeding values for Scots pine populations (0.07-0.22) (Scalfi et al. 2009, García-Gil et al. 2015), attributed the homozygote excess to the presence of null alleles (Scalfi et al. 2009). In our study, only one locus showed evidence of null alleles with a low frequency, therefore this seems unlikely to explain our F_{IS} estimates.

Homozygote excess can be also the result of assortative mating, selection against heterozygotes, the Wahlund effect (subpopulation structure) (Wahlund 1928, García-Gil et al. 2015), or drastic reduction in effective population sizes (Bagnoli & Buonamici 2009). However, as nearby populations obtained very different levels of inbreeding (e.g. 0.196 and 0.046 for CRA and CCC respectively), assortative mating might not be the reason as it is unlikely to occur in adjacent populations because most trees flower at the same time. Furthermore, it seems unlikely to be selection against heterozygotes or a Wahlund effect given that nothing in our data or recent population history point to the existence of unrecognised population substructure. Therefore, the most likely explanation would be a drastic reduction in effective population sizes, as has been previously noted (Mason et al. 2004, González-Díaz et al. 2017)

3.5.3 Geographic diversity gradients and predominant patterns of gene flow

Our results indicated increased nuclear genetic diversity towards the East. This West-East (W-E) trend does not fit theoretical expectations based on inferred patterns of post-glacial colonisation, which for Scots pine, and most other native species in Britain, has occurred from south to north (Birks, 1989). Such a pattern of colonization is expected to leave greater levels of genetic diversity in the south than in the more recently colonised north (Hewitt 1999), and has been found in *Fagus*

sylvatica in Britain (Sjolund et al., 2017), and in other tree species in Ireland (Kelleher et al. 2004) and mainland Europe (King & Ferris 1998; Petit et al., 2002; Cottrell et al., 2005). Equally, the W-E diversity trend does not follow the "central-marginal" hypothesis, which predicts reduced neutral genetic diversity and higher population differentiation towards distribution limits (Eckert et al. 2008). Where the S-N model predicts greater diversity in southern areas, the central-marginal model predicts greater diversity in the centre of the distribution, as populations located further from the centre (even in the south) are less connected. Levels of diversity from our populations, which represent the north-western distribution limit of Scots pine, were similar to those in mainland populations (Naydenov et al. 2011, Provan et al. 1998, Robledo-Arnuncio et al. 2004a), and no signatures of migration were observed (i.e. a S-N or SE-NW trend). Therefore, it may be that the observed W-E diversity trend reflects more recent gene flow process. On the W-E axis, a predominant driver of gene flow in a species with wind-mediated gene dispersal (seed and pollen) is likely to be wind direction. In Scotland the predominant wind direction is west-south-westerly (Dore et al. 2006), and this would seem to be a likely explanation for the observed pattern.

While levels of diversity will be substantially dependent on past population sizes, asymmetric gene dispersal due to prevailing wind direction can also play an important role in shaping the current distribution of genetic diversity (Ennos 1997a). Gene flow patterns were not statistically asymmetric given that gene flow in

Scots pine is extensive and can occur over substantial distances (Lindgren et al. 1995, Robledo-Arnuncio et al. 2004b, 2011). As there is no reason to suggest the existence of geographical or topographical barriers to gene dispersal, gene flow is likely to link nearby populations in any direction. However a strongly dominant wind direction (Dore et al. 2006) would likely favour consistent gene flow from western to eastern populations but not the converse, resulting over time in a gradient of diversity with lower levels in the populations to which gene flow is more restricted. This effect would be reinforced by the fact that western populations are at the very edge of the distribution, with only ocean beyond them, preventing gene input from upwind. The lack of West-East pattern in allele richness might be explained by the diverging trend in some populations, such us CRA, BWR, MEG and ROT, which showed low allele richness but high nuclear diversity. The explanation for this diverging trend might be due to selection or local bottlenecks (i.e. due to a substantial reduction in population size), which are characterized by large losses in allelic richness but only slight decrease of diversity if population size rebounds rapidly (Comps et al. 2001). Since no West-East pattern was observed in chloroplast haplotype diversity, seed dispersal may be more important than pollen in driving any such directionality in gene flow, an implication that tallies with the likely mobility of the two propagule types.

3.6 Conclusions

Native pine forest in Scotland suffered a very substantial historic reduction in abundance. Despite this reduction and the resulting geographical isolation of populations, high levels of genetic variation and low levels of population differentiation persist, suggesting that effective population size, together with extensive gene flow, has been high enough to limit the effects of genetic drift. This finding highlights the importance of maintaining large effective population sizes, especially in geographically marginal populations, to increase the probability of forest persistence (FAO 2014).

Despite potential barriers to gene flow such as population fragmentation (White et al. 2002, Petit & Hampe 2006, Wang et al. 2012, Davies et al. 2013) or phenological asynchrony, gene flow among populations can still be sufficient to counteract their genetic isolation. However a weak signal of isolation by distance was detectable among the Scottish populations, suggesting that some spatial limitation of gene dispersal occurs, although gene flow is extensive. The detected gene flow patterns and geographic distribution of genetic variation were consistent with gene dispersal limitation due to prevailing wind patterns. From a practical point of view, taking into account such landscape impacts on genetic diversity is important when designing afforestation strategies or determining priorities in conservation and management plans.

Although western populations had relatively lower nuclear diversity, and there was greater differentiation and directional bias of gene flow towards the East, there was no evidence to suggest that any of the populations analysed here are genetically at risk. However, over recent decades, there has been extensive establishment of Scots pine plantations throughout the country and it would be interesting to understand the impact such plantations might have on the diversity and structure of subsequent generations of native Scots pine.

3.7 Acknowledgements

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3.8 Supplementary material

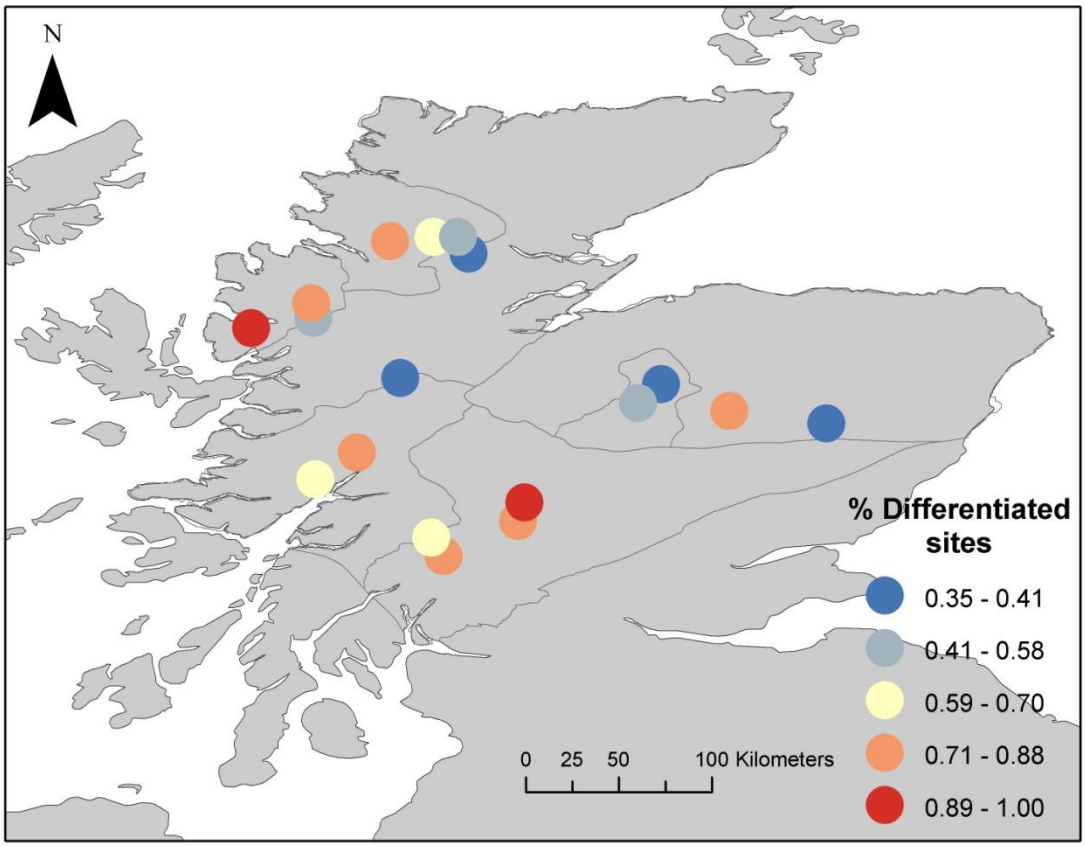
S3.1 Pairwise population differentiation (F_{ST}) (below diagonal) and differentiation index D (above diagonal).

	COG	GLL	CRA	CCC	MEG	BLW	ABE	ROT	ALT	GLT	GLA	AMA	LOC	SHI	BEE	GLE	STO	RHI
COG	-	***0.096***	*0.048*	*0.052*	*0.076*	*0.058*	*0.063*	0.019	*0.074*	0.029	0.021	*0.039*	-0.008	*0.043*	*0.053*	*0.040*	0.024	*0.064*
GLL	**0.034**	-	*0.050*	*0.055*	0.028	*0.049*	0.005	*0.057*	*0.059*	0.020	*0.040*	0.015	*0.079*	***0.085***	*0.041*	*0.066*	*0.063*	*0.071*
CRA	**0.019**	**0.014**	-	0.031	0.021	*0.058*	*0.042*	*0.047*	***0.092***	*0.038*	0.028	0.020	0.037	***0.121***	0.025	*0.051*	*0.073*	*0.087*
CCC	**0.025**	**0.013**	0.007	-	0.026	*0.057*	*0.068*	*0.081*	***0.126***	***0.085***	*0.040*	*0.054*	*0.066*	***0.116***	*0.038*	***0.093***	***0.123***	***0.026***
MEG	**0.035**	**0.016**	0.006	0.013	-	*0.043*	0.014	*0.045*	*0.060*	*0.044*	0.025	*0.045*	*0.077*	***0.127***	0.037	*0.078*	*0.074*	*0.068*
BLW	**0.023**	**0.021**	**0.023**	**0.018**	**0.016**	-	*0.043*	*0.041*	*0.031*	*0.037*	0.023	*0.048*	*0.065*	*0.055*	*0.084*	***0.092***	*0.033*	***0.116***
ABE	**0.022**	0.005	0.012	**0.019**	0.002	**0.015**	-	0.027	*0.039*	0.000	0.014	-0.003	*0.057*	***0.096***	0.029	0.013	0.015	0.019
ROT	0.007	**0.021**	**0.022**	**0.028**	**0.019**	**0.011**	0.009	-	0.012	-0.012	0.010	0.015	0.014	*0.062*	***0.085***	0.033	*0.012*	*0.052*
ALT	**0.029**	**0.026**	**0.044**	**0.045**	**0.029**	**0.013**	**0.018**	0.006	-	0.018	0.020	0.035	***0.074***	*0.070*	***0.123***	***0.090***	*0.030*	***0.091***
GLT	0.009	0.005	**0.014**	**0.025**	**0.017**	**0.012**	0.002	-0.005	0.008	-	0.007	0.003	0.021	*0.064*	*0.053*	0.010	-0.001	0.034
GLA	0.006	0.010	**0.015**	0.011	**0.017**	0.009	0.008	0.007	0.010	0.003	-	0.011	*0.040*	*0.048*	*0.059*	0.021	0.022	*0.061*
AMA	**0.015**	0.005	0.012	**0.015**	**0.018**	**0.013**	-0.001	0.005	**0.013**	0.004	0.002	-	0.023	*0.069*	0.040	0.016	0.033	0.024
LOC	-0.001	**0.022**	0.010	**0.023**	**0.026**	**0.024**	**0.014**	0.003	**0.032**	0.005	**0.016**	0.007	-	*0.064*	0.029	0.029	0.026	*0.056*
SHI	**0.023**	**0.036**	**0.053**	**0.046**	**0.065**	**0.028**	**0.046**	**0.031**	**0.038**	**0.032**	**0.019**	**0.029**	**0.035**	-	***0.132***	***0.095***	*0.049*	***0.135***
BEE	**0.025**	**0.014**	0.012	**0.021**	**0.020**	**0.040**	0.009	**0.033**	**0.050**	**0.020**	**0.026**	**0.017**	**0.012**	**0.067**	-	*0.042*	*0.077*	*0.061*
GLE	**0.018**	**0.019**	**0.017**	**0.031**	**0.031**	**0.041**	0.004	**0.020**	**0.046**	0.009	**0.013**	0.006	0.009	**0.048**	0.010	-	0.021	*0.047*
STO	0.009	**0.022**	**0.024**	**0.042**	**0.026**	**0.014**	0.001	0.004	**0.017**	0.001	0.011	0.008	0.006	**0.027**	**0.024**	0.007	-	*0.064*
RHI	**0.027**	**0.023**	**0.029**	**0.033**	**0.028**	**0.041**	0.007	**0.020**	**0.036**	**0.015**	**0.025**	0.008	**0.017**	**0.057**	**0.013**	**0.016**	**0.019**	-

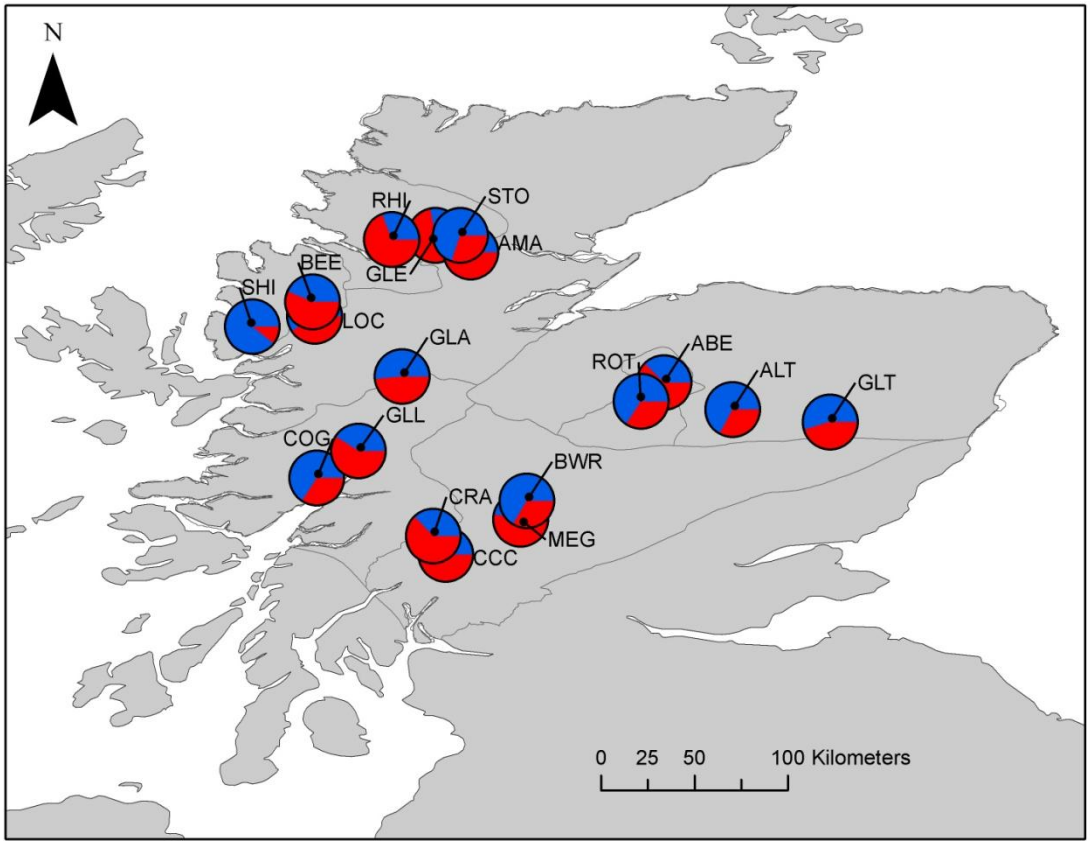
Numbers in bold indicate significant *P*-values P<0.05.

For the Differentiation index D, numbers in bold indicate significant *P*-values P<0.05 after Bonferroni correction, and number in italics indicate significant *P*-values P<0.05 without Bonferroni correction. P-values for the differentiation index D were obtained by bootstrapping 10,000 times.

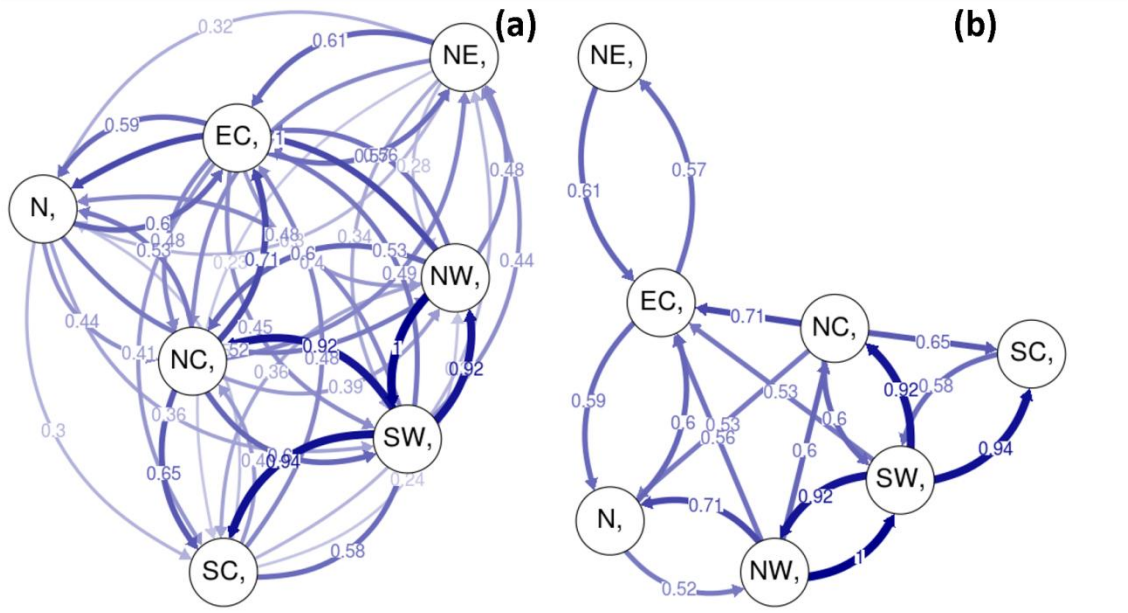
S3.2 Percentage of differentiated sites within Scotland using nSSR



S3.3 Number of genetic clusters (K=2) identified by STRUCTURE for nSSR.



S3.4 Relative migration networks for nSSR with populations sorted in seed groups. Seed group codes correspond to the following group of populations (see Table 2 for population details): NE= ALT, GLT; SC= CCC, MEG, BWR; EC= ABE, ROT; SW= COG, GLL, CRA; NC= GLA, AMA; NW= LOC, SHI, BEE; N= GLE, STO, RHI. (a) No using a threshold, (b) using a threshold of 0.5.



Chapter 4

Ecology and management history drive spatial genetic structure in Scots pine

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Photos in Chapter 4 main page taken from; left: http://www.forestry-memories.org.uk and right: L. Matías.

4.1 Abstract

Forest management practices that remove trees from stands can promote substantial changes in the distribution of genetic diversity within and among populations at multiple spatial scales. In small and isolated populations, elevated inbreeding levels might reduce fitness of subsequent generations and threaten forest resilience in the long term. Comparing fine-scale spatial genetic structure (SGS) between life stages (e.g. adult and juvenile cohorts) can identify when populations have undergone disturbance, even in species with long generation times. Here, we studied the effects of historical and contemporary forest management, characterized by intense felling and natural regeneration respectively, on genetic diversity and fine-scale SGS in adult and juvenile cohorts. We examined fragmented Scots pine (*Pinus sylvestris* L.) stands in the Scottish Highlands, and compared them with a remote, unmanaged stand. A total of 777 trees were genotyped using 12 nuclear microsatellite markers. No difference was identified in allelic richness or gene diversity among stands or life stages, suggesting that historical and contemporary management have not impacted levels of genetic variation. However, management appears to have changed the spatial distribution of genetic variation. Adult genotypes from managed stands were more spatially structured than in the unmanaged stand, a difference mediated by contrasts in tree density, degree of fragmentation of stands at the time of establishment and rate of gap creation. Surprisingly, juveniles were less spatially structured than adults in the managed stands, suggesting an historical erosion of the structure of the adult cohort but contemporary recovery to natural dynamics, and

indicating a high capacity of the species to recover after disturbance. Here we showed that using the spatial component of genetic diversity can help to detect both historical and contemporary effects of disturbance in tree populations. Evaluation of successional change is important to adequately detect early responses of tree populations to forest management practices. Overall, our study suggests that combining sustainable management with forest conservation practices that ensure larger effective population sizes is key to successfully maintaining genetic diversity in Scots pine.

4.2Introduction

A prolonged history of forest exploitation based on the harvesting of trees has resulted in widespread modification of Europe's forests, impacting genetic diversity within and among populations (FAO, 2014). Currently, over 70% of European forests (representing some 15% of European forest area) are subject to a management plan or its equivalent (Forest Europe, 2015) but, despite a substantial shift toward sustainable practices over the past 25 years (FAO, 2015), the consequences of historical management practices such as extensive felling on the distribution of genetic diversity in tree species remain largely uncertain. Genetic diversity plays an essential role in underpinning forest resilience by facilitating evolutionary processes, and it is key in forest responses to disturbances, such as habitat loss, fragmentation or pathogen attack (Schaberg et al., 2008; Cavers and Cottrell, 2014). Consequently, understanding how historical and contemporary forest management have shaped patterns of genetic diversity allows evaluation of the potential resilience of European forests and informs the development of adaptive management plans.

The impact that tree removal can have on population genetics has been addressed through exploration of levels of neutral genetic variation, revealing changes in gene frequencies (Schaberg et al., 2008) and loss of alleles (Adams et al., 1998; Rajora et al., 2000; Kettle et al., 2007; Ortego et al., 2010), yet many studies have failed to detect significant effects (Bradshaw, 2004; García-Gil et al., 2015; Rajora and Pluhar, 2003; Schaberg et al., 2008; Young et al., 1996). Some authors attribute the lack of effect to

the long generation time in trees, because changes in genetic diversity after disturbance may take many generations (Lowe et al., 2005). However, changes in tree distribution and age structures can alter the spatial organisation of genetic variation, even when overall levels of variation are maintained, allowing us to explore the genetic legacy of forest management (Piotti et al., 2013; Sjölund and Jump, 2015).

In naturally regenerated tree populations, genotypes are not distributed randomly. Typically, individuals become less genetically similar as the distance between them increases (Jump and Peñuelas, 2007; Paffetti et al., 2012; Vekemans and Hardy, 2004), causing a phenomenon known as spatial genetic structure (SGS). Restricted dispersal results in offspring being more likely to establish close to the mother tree (Jump et al., 2012; Pandey et al., 2012). Consequently, the pollen and seed dispersal mechanism will strongly influence the extent and magnitude of SGS within a species. For example, plants with animal dispersed pollen usually show greater SGS than those with wind dispersed pollen (Vekemans and Hardy 2004). Furthermore, individual density is usually inversely correlated with SGS. For example, the extent of SGS in low density populations of *Acer pseudoplatanus* is nine times greater than in high density populations (Vekemans and Hardy 2004).

The ecological determinants of SGS (such as recruitment frequency, seed and pollen dispersal distance, and individual density) are commonly modified by forest management practices that remove trees. Consequent changes in SGS may alter local

mating patterns and the distribution of genetic diversity in subsequent generations (Smouse and Peakall, 1999). Furthermore, different forest management practices, such as felling, coppicing or thinning, will differentially impact selection of individuals and seedling establishment potentially leading to a broad range of genetic impacts (Cottrell et al., 2003; Paffetti et al., 2012; Piotti et al., 2013; Sjölund and Jump, 2015). Distinguishing the effects of forest management on SGS is, therefore, a challenging task.

SGS of plant populations is dynamic and can change across life stages. In individuals that reproduce sexually, seedlings might be affected by compensatory mortality and competitive thinning, post dispersal, thereby altering spatial distribution patterns with age (Ng et al., 2004). Most studies have found greater SGS in early regeneration stages than in mature individuals (González-Martínez et al., 2002; Hardesty et al., 2005; Ng et al., 2004; Soto et al., 2007; Troupin et al., 2006). The successional component of SGS (e.g. comparing SGS between adult and juvenile cohorts) has mainly been studied in order to understand the natural development of SGS (Berens et al., 2014; González-Martínez et al., 2002; Jones and Hubbell, 2006). Such changes in SGS have rarely been used to assess the influence of forest management practices (but see Jones et al., 2006; Leclerc et al., 2015; Troupin et al., 2006).

This study focuses on the remaining fragmented Scots pine (*Pinus sylvestris* L.) forests of the Scottish Highlands (known as Caledonian pine forests), which are believed to be the only native pine forests in the UK. These fragmented remnants

represent a valuable system in which to study the impacts of historical forest management practices because numerous records of management history exist. To understand the effects of historical and contemporary forest management practices, we investigated genetic diversity and fine-scale SGS in adult and juvenile cohorts in two native managed pine forests and compared these with a remote, unmanaged stand. We selected two life stages that were established in distinct periods with contrasting forest management systems: (1) adult trees that established during 19th Century, characterised by high browsing pressure by deer and after a period of intense felling (hereafter historical management); and (2) juveniles that established during the last two decades, characterised by conservation policies promoting natural regeneration (hereafter contemporary management). Specifically we sought to determine: 1) did historical management practice impact genetic diversity and SGS – comparing mature managed and unmanaged stands? and 2) how has contemporary management practice affected diversity and SGS – comparing adults and juveniles from managed stands? We hypothesised that in the absence of effects of historical management, mature managed stands would display similar values of genetic diversity and SGS as those in an unmanaged stand, while in the absence of effects of contemporary management, stronger SGS would be found in the juvenile stages, and similar values of genetic diversity will be evident in both juvenile and adult cohorts.

4.3Material and methods

4.3.1 Study species

Scots pine is a wind-pollinated outcrossing conifer and is the most widely distributed pine species in the world, with a range that spans Eurasia, from the Arctic circle in Norway in the north to the south of Spain and south of Turkey and from the west coast of Scotland to the far east of Russia (Carlisle and Brown, 1968). Populations from southern Europe, Scotland and Asia Minor generally represent isolated occurrences. In Scotland this species occurs at the western limit of its global distribution and constitutes the iconic species of the Caledonian pine forest. Scots pine is typically a pioneer species (together with birch and aspen) that readily regenerates after natural or human disturbances, if competition and grazing pressure are low (Mátyás et al., 2004). It grows well on most soils, nevertheless, due to shade and competition intolerance, it is often restricted to poor soils (Steven and Carlisle, 1959). It is a monoecious species, and female flowering can start at the age of 15 to 30 years, in open to closed stands respectively (Mátyás et al., 2004). Pollen movement is predominantly over tens of metres within a stand (Robledo-Arnuncio et al., 2004b), but it may reach 100 km (Robledo-Arnuncio, 2011). Seeds are primarily wind and gravity dispersed, and typically travel up to 100 metres (McVean, 1963).

4.3.2 Study sites and history of forest management

From a peak distribution around 6,000 years ago, Scots pine in Scotland has been in decline for millennia, with a major retreat 4,000 years ago, initially attributed to a climate shift to wetter conditions (Bennett, 1984), although human and grazing pressures may have also played a significant role (Tipping et al., 2008). The exploitation and reduction in Scots pine extent has been particularly intense from the 18th Century onwards (Hobbs, 2009), mainly characterized by felling and selective logging to provide construction timber (Smout, 2003). The general decrease in forest extent, together with poor natural regeneration in the Caledonian pine forest (due to extensive browsing pressure by deer and sheep), kept this forest at low tree density for many years (McVean, 1963) and has strongly suppressed regeneration during the last 200 years (Steven and Carlisle, 1959). During the last few decades, however, forest management has moved to protect and expand the remaining Caledonian pine forest (Forestry Commission, 2016).

We selected two study sites in Scotland, Abernethy (57°20'N, 3°61'W) and Glen Affric (57°15'N, 5°00'W). Nowadays, these sites constitute some of the largest ancient pine forest in Scotland covering areas of 2452 ha and 1532 ha, respectively (Mason et al., 2004). In each site, an old open native stand was selected, where trees are expected to have been established through natural regeneration of native provenance. Hereafter these stands will be referred to as managed stands. The fire regime in the UK is largely human driven (Davies et al., 2008), but tree mortality

through large fires is uncommon in Scotland. In addition, wind-blow and snow can cause some casualties through the year, and fungi and insects will be minor effects. However, intense forest disturbance in recent centuries can be attributed mainly to forest management practices.

The study site in Abernethy is located at 370 m a.s.l., with south westerly prevailing winds and densities of 160 stems ha^{-1}. Stand composition is formed by Scots pine, with presence of *Juniperus communis*. The understory is predominantly *Calluna vulgaris, Vaccinium myrtillus* and small patches of *V. vitis-idaea*. Historical exploitation at Abernethy has taken place over millennia and high felling and browsing pressure during the 18th Century are reflected in the progressive contraction of the extent of Abernethy forest in historical maps from 1750 until 1830 (Smout et al., 2005, Summers et al. 2008). By 1858, the forest was represented only by scattered trees in the study site and followed by enclosure of the forest as deer forest occurred in 1869 (O'Sullivan, 1973). In the 1980s the area was designated a National Natural Reserve. Seasonal grazing by sheep was stopped in 1990 and deer fences were removed (Beaumont et al., 1995). Since then, culling of deer has kept the population stable and compatible with forest regeneration. By 1992 the percentage of seedlings with evidence of browsing had fallen from 72% to 43% with an increase of 20% in the number of established seedlings and saplings (Beaumont et al., 1995).

The study site in Glen Affric is located at 260 m a.s.l., south west of Loch Affric, where the prevailing winds are south westerly, and stand density is 103 stems ha^{-1}.

Stand composition is dominated by Scots pine and the vegetation layer is predominantly *C. vulgaris* with small patches of *V. vitis-idaea* and *V. myrtillus*. Evidence from pollen records from west Glen Affric, where our stand is located, show a sustained low tree cover around these sites for several thousand years as a result of prolonged human impact, with the recent expansion of the forest when the present tree cohort developed around 1880 (Shaw, 2006). Historical documents report felling of trees during the 18th and 19th Centuries (Smout et al., 2005) with the decline evident in pollen records. Following a period of intensive sheep and deer grazing in the late 20th Century a major effort was made to protect and restore the remaining native pine forest (Bain, 2013). Glen Affric was initially declared as a Caledonian Forest Reserve in 1961 by the Forestry Commission (Bain, 2013) and later, in 1984, a National Natural Reserve.

To compare our heavily managed stands with an unmanaged case, and since unmanaged stands do not exist in Scotland, pre-existing samples from a boreal site in Western Siberia were used (60°54'N, 68°42'E). These samples were taken from within a continuous population with extensive areas of natural forest, with a stand density of 470 stems ha^{-1}. These forests have never been altered by humans, but are subject to regular natural disturbance by fire on roughly 50 year timescales. In these boreal forests, competition forces Scots pine to forest edges and onto poor quality sites such as sandy soils or bogs, and it is outcompeted on better soils by *Pinus*

sibirica, *Larix sibirica* and *Populus tremula*. As a result even mature individuals may be small. Hereafter this stand will be referred to as the unmanaged stand.

In Scots pine, genetic variation is partitioned predominantly within rather than among populations, and levels of within-population genetic diversity across the range of Scots pine are similarly high (Wachowiak et al., 2014, 2011). Previous studies of diversity across the range of this species have shown that genetic differentiation among even distant populations of Scots pine is low (Naydenov et al., 2007; Provan et al., 1998; Prus-Glowacki and Stephan, 1994; Wang et al., 1991) but see (Forrest, 1982; Prus-Glowacki et al., 2012). Some authors attribute this homogeneity to common ancestry, as well as extensive gene flow (Chybicki et al., 2008) and lack of major physical barriers (Naydenov et al., 2007). As absolute genetic diversity levels in the managed and unmanaged stands are of similar magnitude, and the physical capacity for gene movement should be similar in each, we can assume that the primary driver of genetic structure will have been the presence or absence of significant human intervention. Therefore, this comparison can be informative regarding the processes that are likely responsible for the observed spatial pattern of genetic diversity at fine scales.

4.3.3 Sample collection, life stages and stand structure

We selected stands of 200 m × 200 m in Abernethy and Glen Affric, respectively. Sampling strategy was designed to account for short distance classes in order to

detect fine-scale SGS, choosing individuals randomly and assuring sufficient numbers of pairwise comparisons in each distance class, as recommended by Cavers et al. (2005). We sampled needles from two life stages, juveniles and adults. Sample size per stand in each life stage varied from 131 to 181 (Table 1). All individuals were mapped using a GARMIN 62s handheld GPS and diameter was measured at breast height (d.b.h.). The d.b.h. was used as a proxy of age, defining juveniles as individuals with d.b.h. below 10 cm. Existing data from trunk cores from nearby adult pines in Abernethy (Summers et al., 2008) were used to calibrate the relationship between d.b.h. and age.

The unmanaged study site was sampled in three sub-stands of 50 x 50 m along a linear transect of 300 m, which were combined to give a single stand sample for subsequent analysis. All sampled individuals were mapped, measured for d.b.h. and tree height classified as short (<2m) or tall (>2m). Juveniles were defined as short individuals with d.b.h. below 10 cm. Sample size in each life stage varied from 57 to 73 (Table 1). Thirty random trunk sections from adult pines were taken from the unmanaged site to calibrate the d.b.h.-age relationship. We evaluated the relationship between d.b.h. and tree age, and whether this relationship varied among sites using a linear model in R 3.0.1 (R Core Team, 2013). We chose d.b.h. as the response variable and tree age and site (Abernethy and unmanaged) were the predictor variables. The interaction between the predictor variables was tested and

compared with a model without interactions by using the Akaike Information Criterion.

4.3.4 Microsatellite analyses

Total genomic DNA was extracted from 50 mg silica gel dried needles using QIAGEN DNeasy 96 Plant Kit (QIAGEN Ltd. Crawley, UK) following the manufacturer's protocol. All individuals were genotyped at twelve nuclear microsatellite markers (SSR): psyl2, psyl16, psyl17, psyl36, psyl42, psyl44, psyl57 (Sebastiani et al., 2011), SPAC7.14, SPAC12.5 (Soranzo et al., 1998), PtTX4001, PtTX4011 (Auckland et al., 2002) and SsrPt_ctg4698 (Chagné et al., 2004), combined in two multiplexes of six loci each. Multiplex 1 consisted of primers psyl2, psyl17, psyl42, psyl44, PtTX4001 and PtTX4011 at concentrations of 3 μl, 2 μl, 2 μl, 2 μl, 3 μl and 2 μl respectively. Multiplex 2 consisted of primers psyl16, psyl36, psyl57, SPAC7.14, SPAC12.5 and SsrPt_ctg4698 at concentrations of 2 μl each. Reactions were carried out in a final volume of 10 μl with 1X of QIAGEN Type-it Multiplex PCR Master Mix, 1 μM of each multiplex and 25 ng of template DNA. Annealing temperature for both multiplexes was 56°C. Polymerase chain reactions (PCR) were performed in Veriti™ Thermal cycler (Applied Biosystems, Bleiswijk, Netherlands), with the following programme: 1 cycle at 95°C for 4 min followed by 35 cycles at 95°C for 45 s, 56°C for 45 s, 72°C for 45 s, and a final step at 72°C for 5 min. PCR products were analysed by DNA Sequencing and Services, Dundee, UK, using an

Applied Biosystems 3730 DNA Sequencer with reference to a LIZ 500 size standard. Fragment analysis results were scored using GENEMARKER V.2.6.0. (SoftGenetics, State College, PA, USA). FLEXIBIN (Amos et al., 2007) was used to check discrete classes of raw allele sizes and MICRO-CHECKER (Van Oosterhout et al., 2004) to check genotyping errors and null allele frequencies. Several markers showed evidence of null alleles at very low frequencies (maximum frequency of 0.04, data not shown), which is far below to the threshold at which null alleles can result in a significant underestimate of expected heterozygosity, estimated as 0.2 (Belletti et al., 2012; Chapuis and Estoup, 2007). Therefore, all markers were kept for further analysis.

4.3.5 Genetic diversity and spatial genetic structure analysis

Genetic diversity estimators within stands and life stages were estimated using FSTAT 2.9.3.2 (Goudet, 1995): mean number of alleles per locus (A), rarefied allelic richness (A_R) (rarefied to 57 individuals for each stand and life stage), expected heterozygosity (H_E) and inbreeding coefficient (F_{IS}). We conducted ANOVAs to test for differences in A, A_R, and H_E between stands and life stages in R 3.0.1 (R Core Team 2013). We calculated F_{ST} among stands and life stages in ARLEQUIN v3.5 (Excoffier and Lischer, 2010), and the differentiation index D (Jost, 2008) implemented in the R package DEMEtics (Gerlach et al., 2010). In both cases, significance values were determined for a 5% nominal level after Bonferonni

correction. F_{ST} estimates differences in allele frequencies among stands, whereas differentiation index *D* measures the fraction of allelic variation among them.

We implemented fine scale SGS analyses in SPAGeDi 1.4b (Hardy and Vekemans, 2002). In order to test for significance in genetic relatedness, the Kinship coefficient of Loiselle et al. (1995) (F_{ij}) was estimated as $F_{ij}=(Q_{ij}-Q_m)/(1-Q_m)$, where Q_{ij} is the probability of identity in state for random gene copies from two individuals *i* and *j*, and Q_m is the average probability of identity by state for gene copies coming from random individuals from the sample. A regression between the Kinship coefficient F_{ij} and the logarithm of pairwise geographic distances of individuals was computed (b_F). Standard errors of the regression slope were computed using a jackknife procedure over loci. The significance of the slope of the regression was tested using 10,000 permutations of locations among individuals. To visualize the SGS, we plotted average pairwise estimates of genetic relatedness as a function of distance to generate spatial autocorrelograms. Analyses were conducted for each stand and life stage separately across the full distance range. SGS_{MAX} was also calculated for each stand and life stage, which is the greatest distance at which the Kinship coefficient of a given distance class *F(d)* is significant at $p<0.05$ (Jump et al., 2012). We also calculated the *Sp* statistic, as suggested by Vekemans and Hardy (2004), to allow comparability among stands and life stages with other studies. The *Sp* statistic was determined as $-b_F/(1 - F_1)$, where b_F is the regression slope of kinship coefficient estimate (*F*) on

distance classes and F_1 is the kinship coefficient for adjacent individuals in the first distance interval.

Because the number of pairs within each distance class should ideally exceed 50 pairs of individuals, we set the distance intervals of at least 10 metres (Cavers et al., 2005; Jump and Peñuelas, 2007). Overall, we established 10 distance classes for the managed stands (0-10, 10-20, 20-30, 30-40, 40-50, 50-60, 60-70, 70-80, 80-90, 90-100), and 8 distances classes in the unmanaged stand (0-10, 10-20, 20-30, 30-60, 60-70, 70-80, 80-90, 90-100). Distance classes between 30 and 60 metres were combined in the unmanaged stand to ensure sufficient numbers of pairs in the class. We also tested other distance class options and longer final distances up to 200 metres, and found they revealed similar and no more informative results. In addition, in the unmanaged stand, analysis of each sub-stand was also conducted separately, and the same results were obtained.

4.4Results

4.4.1 Stand structure

Tree diameter distribution for managed stands was bimodal, with the highest frequencies for juvenile individuals at diameters between 0 and 10 cm (Fig. 4.1). A gap of adult individuals with diameter classes between 10 to 30 cm and 10 to 25 cm occurred in Abernethy and Glen Affric, respectively (Fig. 4.1). Contrastingly, tree diameter distribution in unmanaged stand was more skewed towards smaller

diameters. There was no gap in the distribution in this case, instead there was a gradual decrease in the numbers of individuals with increasing diameter class (Fig. 4.1).

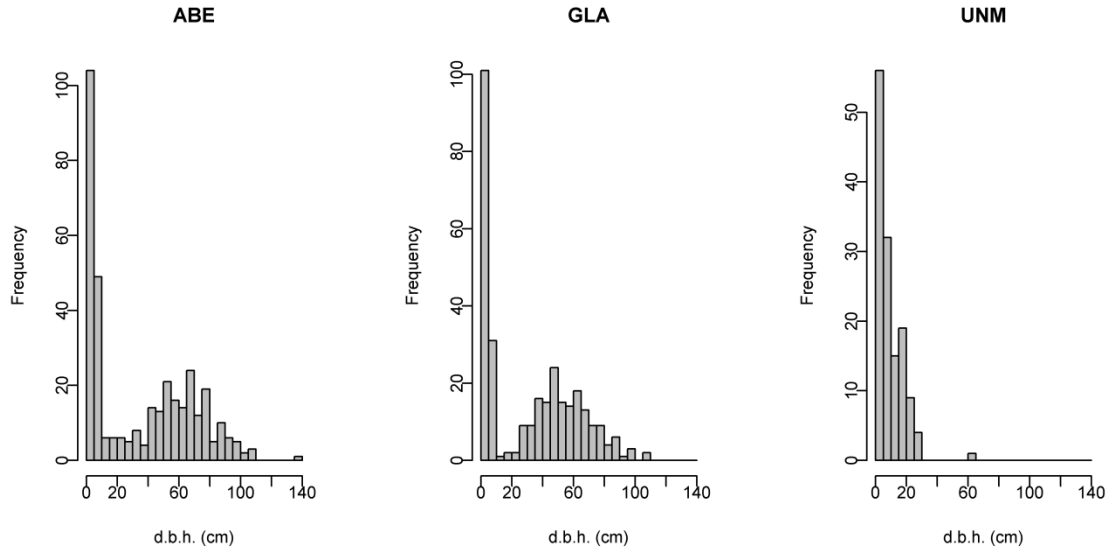

Figure 4.1 Tree diameter (d.b.h.) distribution in the three study sites: Abernethy (ABE), Glen Affric (GLA) and the unmanaged site (UNM). Juvenile stem diameter was measured at 10 cm height. Data are presented in intervals of 5 cm.

We found that d.b.h. was dependent on age and site (F=29.85, R^2=0.31), showing strong differences among age (t=3.81, p<0.001), and among sites (t=-6.03, p<0.001). However, we did not find significant interactions between age and study site (Fig. 4.2). The relationship between d.b.h. and age suggested that differences in age profiles in the two sites were smaller than differences in tree size (e.g. trees with different d.b.h. could have a similar age).

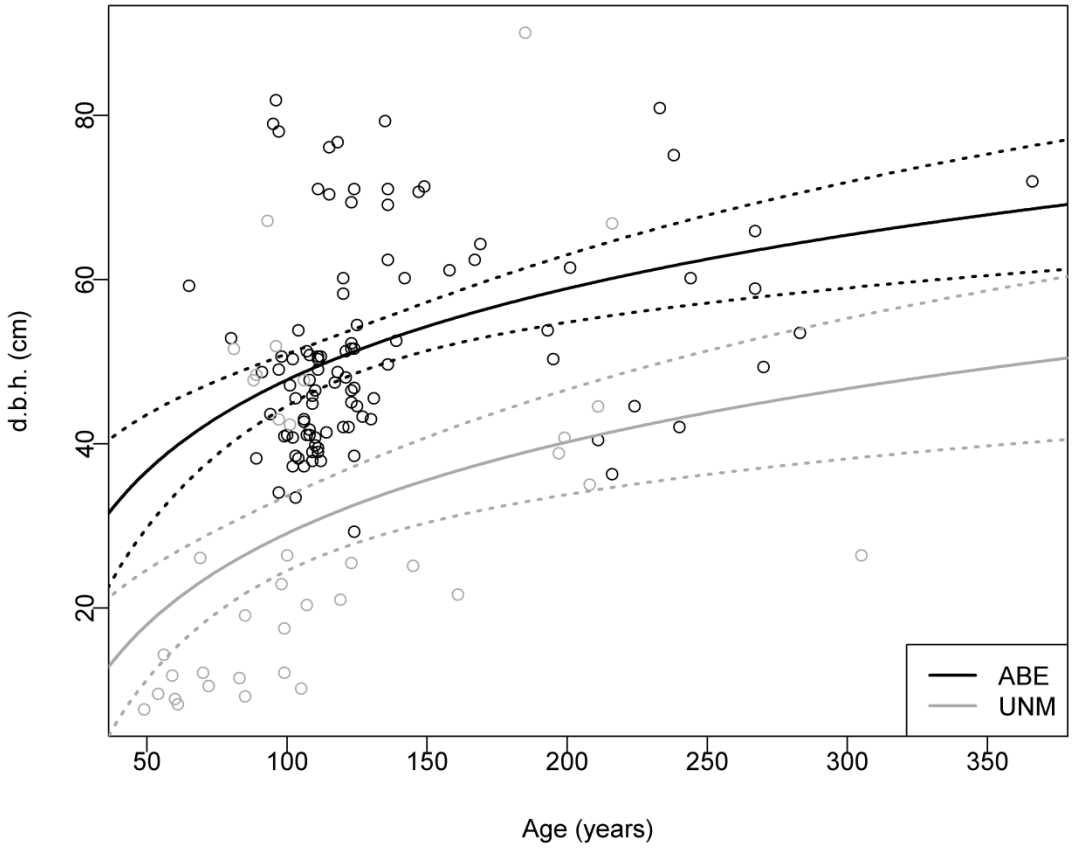

Figure 4.2 Relationship between d.b.h. and age for the managed site of Abernethy (ABE) and the unmanaged site (UNM). Lines of best fit are represented by solid lines and 95% CI by dashed lines. Dots represent observed values.

4.4.2 Genetic diversity

Genetic diversity parameters did not significantly differ between managed and unmanaged stands (Table 4.1). Among the twelve nuclear loci analysed, the number of alleles (*A*) in the managed stands ranged from 3 to 31 and 4 to 29 per locus for Abernethy and Glen Affric respectively for both life stages combined (multilocus average of 9.92 for each site). *A* ranged from 3 to 31 in the unmanaged stand, with a multilocus average of 9.83 again for both life stages combined. For rarefied allele richness (A_R) in the managed stands, multilocus estimates obtained mean values of

8.99 and 8.83 for Abernethy and Glen Affric respectively and 8.95 for the unmanaged stand both life stages combined, based on a minimum number of 126 individuals. Expected heterozygosity levels (H_E) showed multilocus estimates of 0.58 in Abernethy and 0.56 in Glen Affric, and similar values of 0.58 for the unmanaged stand for both life stages combined (See supplementary material S4.1 for genetic diversity estimators on each site and life stage and for detailed information of each microsatellite). Neither A, A_R or H_E significantly differed between managed vs. unmanaged stands (all p-values > 0.05). However, some differences appeared in the inbreeding coefficient (F_{IS}) which was significant and higher for both managed stands, indicating significant departure from Hardy–Weinberg equilibrium, whereas it was not significant for the unmanaged stand (Table 4.1). F_{ST} values indicated low but significant differences among the two managed stands (F_{ST}=0.004, p<0.001), and higher differences when comparing them with the unmanaged stand (F_{ST}=0.03 and F_{ST}=0.04, p<0.001, for Abernethy vs. unmanaged and Glen Affric vs. unmanaged respectively), indicating that despite remarkably similar overall levels of genetic diversity, their genetic composition differs to some extent.

When comparing life stages within stands, neither A, A_R or H_E significantly differed (all p-values > 0.05). F_{ST} values indicated no significant differences among life stages in Abernethy and the unmanaged stand, however low but significant F_{ST} occurred among life stages in Glen Affric. In agreement, differentiation index D showed the

same pattern, although values were consistently larger (See supplementary material, S4.2).

Table 4.1: Summary of multilocus genetic diversity and SGS estimators for each study site and life stage.

Study site	Life stage		Genetic diversity estimators				Spatial genetic structure estimators			
		N	*A*	A_R	H_E	F_{IS}	$F_{(1)}$	SGS_{MAX} (m)	$b_F \pm$ SE	*Sp* ± SE
Abernethy	Adult	181	9.50	7.11	0.587	0.052***	0.0291***	20	-0.0044 ± 0.0006***	0.0045 ± 0.0028
	Juvenile	170	9.25	6.72	0.583	0.080***	0.0183***	18	-0.0028 ± 0.0009**	0.0029 ± 0.0023
Glen Affric	Adult	165	8.92	6.79	0.568	0.063***	0.0298***	40	-0.0097 ± 0.0023***	0.0098 ± 0.0010
	Juvenile	131	9.25	6.74	0.561	0.049**	0.0156***	20	-0.0118 ± 0.0027***	0.0119 ± 0.0006
Unmanaged	Adult	57	7.58	6.51	0.576	0.012	-0.0033	0	0.0006 ± 0.0005	-0.0006 ± 0.0005
	Juvenile	73	8.17	6.94	0.582	0.021	0.0067	5	-0.0017 ± 0.0010*	0.0018 ± 0.0011

N, sample size; *A*, mean number of alleles per locus; A_R, rarefied allelic richness; H_E, expected heterozygosity; F_{IS}, inbreeding coefficient. $F_{(1)}$, Kinship coefficient for first distance class (0-10m); SGS_{MAX}, greatest distance at which the Kinship coefficient of a given distance class *F(d)* is significant at $p<0.05$; $b_F \pm$ SE, regression slope of the Kinship coefficient *Fij* computed among all individuals against geographical distances ± standard error; *Sp* ± SE, *Sp* statistic ± standard error. Significant *P*-values are indicated as $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. *P*-values for F_{IS} are obtained after 10,000 permutations of gene copies within individuals of each stand.

4.4.3 Spatial genetic structure

We found significant SGS in all managed stands for both life stages which extended up to 40 metres further than the unmanaged stand (Table 4.1 and Fig. 4.3). The kinship coefficient for the first distance class $F_{(1)}$ and the *Sp* statistic also reflected the relationship between the extent of SGS and historical management, which was larger for managed than for unmanaged stands (Table 4.1).

When comparing SGS among life stages within stands, both SGS_{MAX} and $F_{(1)}$ were larger for adult than for juvenile stages in the managed stands (e.g. SGS_{MAX} extended up to 20 metres further in adults than juveniles) (Table 4.1 and Fig. 4.3). In contrast, SGS was larger for juveniles than for adults for the unmanaged stand, with significant SGS only at distances of less than 10 metres in the juvenile stage (Table 4.1 and Fig. 4.3). In the managed site of Glen Affric, we found that at 50-80 m trees were less genetically similar than expected compared with a random distribution of genotypes (see significant negative values of Kinship coefficient at distances between 50 and 80 metres in Glen Affric in Fig. 4.2). The minimum number of pairwise comparisons per distance class in the managed stands for each life stage was 106 individuals, whereas it was 63 individuals in the unmanaged stand. The *Sp* values did not reflect the same relationship between the extent of SGS with contemporary management as SGS_{MAX} and $F_{(1)}$ did. Thus, of the managed stands, *Sp* value was not significantly different between adults and juveniles in Abernethy, whereas it increased from adults to juveniles in Glen Affric (Table 4.1).

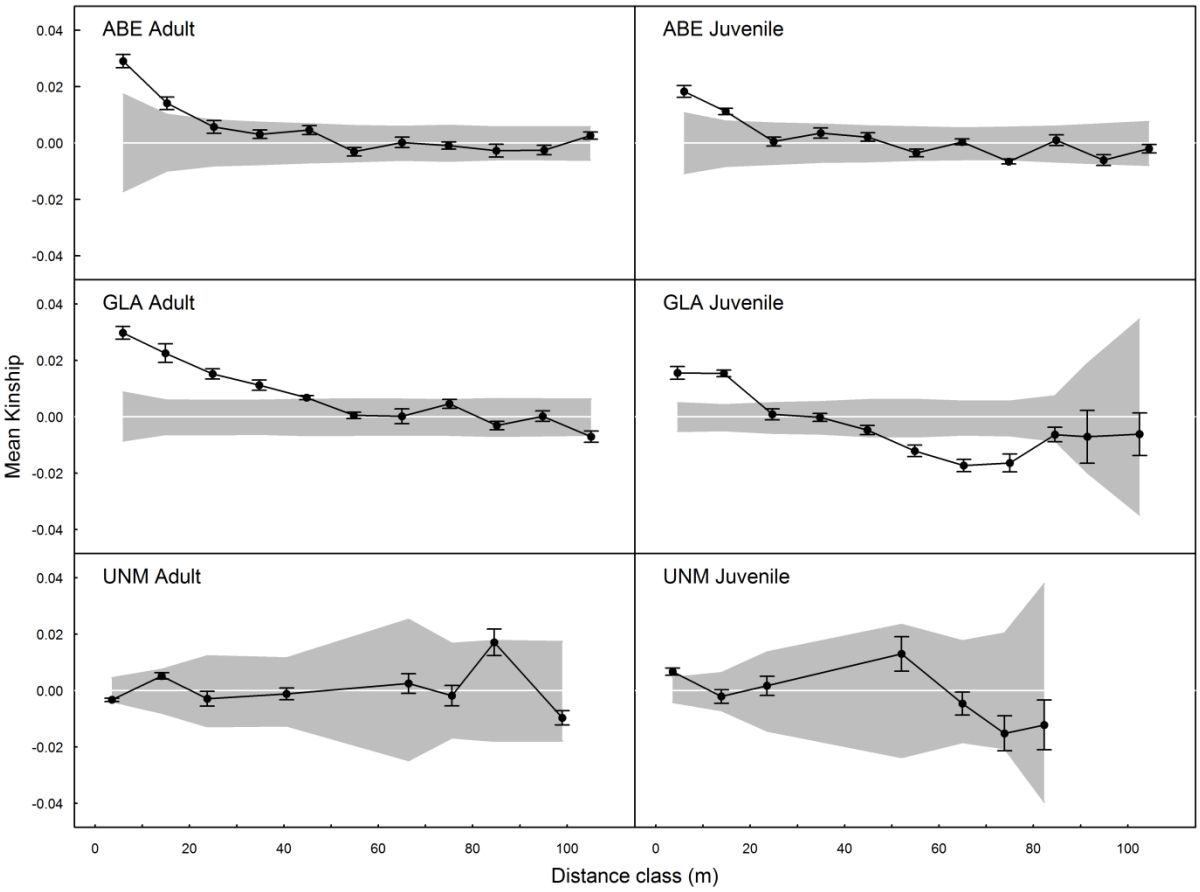

Fig. 4.3: Spatial autocorrelograms for each study site: Abernethy (ABE), Glen Affric (GLA) and the unmanaged site (UNM); and life stage (adult and juvenile) based on the kinship coefficient F_{ij}, estimated from 12 microsatellite loci, and consecutive 10 m distance classes (note that for the unmanaged stand distance classes were combined between 30 to 60 metres). Shaded areas represent 95% confident intervals obtained from 10,000 permutations of genotypes among locations. Black bars around mean F_{ij} values represent standard errors derived through jackknifing over loci.

4.5Discussion

We found two main results: 1) although overall levels of genetic diversity were strikingly similar, more extensive spatial structuring of genetic diversity was found in the mature managed stands when compared with the unmanaged one; 2) in contrast to expectations, a reduced extent of spatial genetic structure was found in the early stages of regeneration of the managed stands compared with the adult

cohorts, again despite no difference in overall levels of genetic diversity between life stages. These patterns suggest that both historical and contemporary management can significantly alter spatial genetic structure of Scots pine. Here, we combine ecological information with historical data on the stands to better understand the mechanisms that are likely responsible for these differences in spatial genetic structure.

4.5.1 Impact of historical forest management practices

Notable differences in size profiles appeared between managed and unmanaged stands, (e.g. mean d.b.h. generally bigger in managed stands (Fig. 4.1)). However, the d.b.h.-age relationship was different among managed and unmanaged stands (Fig. 4.2), linked to the growth-retarding effect of the bog habitat of the latter. Hence, the contrast in age profiles –a more important comparison for SGS analysis– was much smaller than for size profiles (e.g. small trees from the unmanaged stand often had similar ages to much larger trees from the managed one). The age profile of the stands was strongly reflective of their distinct histories, with large, old trees present in the managed sites plus a pulse of recent regeneration, whilst a much wider range of ages was present in the unmanaged one, with fewer very old trees. The structure in the unmanaged site is likely to reflect the natural fire disturbance dynamics to which it is exposed. These dynamics are likely in turn to affect genetic structure, with a higher turnover in the unmanaged stand –shown by the diverse, but generally

young age profile– indicating a higher potential for gene dispersal and therefore erosion of spatial structure.

Genetic diversity of both mature managed sites, as indicated by allelic richness and expected heterozygosity, did not differ significantly from the unmanaged stand but instead was remarkably similar (e.g. H_E: 0.57-0.59 vs. H_E: 0.58, respectively). Although average diversity levels were lower than those reported in mainland European populations of Scots pine using nuclear SSR (H_E: 0.62-0.85) (Scalfi et al. 2009; Naydenov et al. 2011; Nowakowska et al. 2014; García-Gil et al. 2015) differences might be explained by the number of markers used and their specific levels of polymorphism. Thus, for example, selecting two of the three markers used by Scalfi et al. (2009), SPAC 7.41 and SPAC 12.5, the mean value of genetic diversity in our study (0.9) would be higher than previously reported. Also, the markers with the lowest values of diversity in our study, psy144 and psy12, had very similar low values in mainland European populations (Sebastiani et al., 2011) (see supplementary material S4.1). Previous studies in Scottish populations of Scots pine have also reported relatively high levels of genetic variation using other molecular markers (Forrest, 1982, 1980; Kinloch et al., 1986; Provan et al., 1998; Sinclair et al., 1998; Wachowiak et al., 2013, 2011).

High levels of genetic variation at the population level suggests that effective population size has been sufficiently high to restrict effects of genetic drift despite

intensive management and geographical isolation of populations. Scots pine is a wind-pollinated tree with wind-dispersed seed, and achieves high levels of gene flow by: (1) long seed wings, up to four times as long as the seed (Steven and Carlisle, 1959), (2) low seed mass (Castro, 1999) (here 2.9 to 12.64 mg), on average smaller than other pine species (9.1 to 233 mg) (Vander Wall, 2003), and (3) extensive pollen flow, from 17-22 m (Robledo-Arnuncio et al., 2004b) and up to 100 km in small fragments (Robledo-Arnuncio, 2011) (similar to other wind-pollinated tree species). Therefore, it appears that gene flow has been sufficient to prevent erosion of genetic diversity. F_{IS} values, an indirect measure of inbreeding, were not high in the managed sites although they were significantly higher than in the unmanaged site (0.05-0.06 vs. 0.01 respectively), suggesting that although gene flow has prevented loss of genetic diversity at the population level, fine scale alterations to gene flow might have taken place. Drastic reduction of population sizes can induce higher rates of selfing and mating between relatives (Robledo-Arnuncio et al., 2004a). The small size of the population at the time of establishment of the current adult cohorts, as indicated by historical data (Shaw, 2006; Summers et al., 2008), might explain this pattern.

Consistent differences in SGS were found in the mature managed stands which showed greater extent and magnitude of structure, as indicated by SGS_{MAX} up to 40 metres and higher $F_{(1)}$, compared with the unmanaged one. The extent of SGS of the mature managed stands was also larger than the values reported for Scots pine

(Chybicki et al., 2008) and other *Pinus* species, which typically had values below 15 metres (De-Lucas et al., 2009; González-Martínez et al., 2002; Jones et al., 2006; Marquardt and Epperson, 2004; Parker et al., 2001; Troupin et al., 2006; Williams et al., 2007). It should be noted, however, that SGS estimates in Parker et al. 2001 and Jones et al. 2006 were based on allozyme markers, and the need for caution when comparing SGS extent with different molecular markers has been previously highlighted (Jump and Peñuelas, 2007).

Values of SGS extent more comparable to those in our managed stands were observed in fragmented populations of *Pinus pinaster* (~ 20 metres) (De-Lucas et al., 2009). The high values of SGS_{MAX} in the managed stands are likely a consequence of the drastic reductions in the number of seed and pollen donors, which are two of the main drivers of SGS (e.g. due to felling practices). The larger extent of SGS observed in Glen Affric may arise from local differences in historical management, with a prolonged limited tree cover due to human activities (Shaw, 2006), which is also reflected in the lower density of the site. The very short spatial scale of genetic structure in the mature unmanaged stand was remarkably similar to that in undisturbed continuous populations of *P. pinaster* which displayed either weak or no relatedness, with maximum values of SGS_{MAX} of 10 metres (De-Lucas et al. 2009). As these populations have contrasting local contexts, the unmanaged stand being part of the extensive core Eurasian population whereas the undisturbed population of *P. pinaster* being a distributional edge population, the similarity in SGS values observed

seems likely to be due to their common unmanaged state. Therefore, it seems clear that tree density, degree of fragmentation of stands at the time of establishment and rate of gap creation play a major role in the formation of SGS in populations. *Sp* values for the mature managed stands (0.0045 and 0.0098) were remarkably higher than for the non-managed stand (-0.0006). Similarly, De-Lucas et al. (2009) found differences in the *Sp* values between fragmented and continuous populations of *P. pinaster*, although they were generally higher than the values reported in this study.

4.5.2 Impact of contemporary forest management practices

In the managed stands, there were no differences among life stages in the levels of allelic richness or gene diversity, suggesting contemporary management has not impacted genetic variation. However, we found higher relatedness – as higher SGS intensity and extent – in adults than in juveniles, with a greater discrepancy in the Glen Affric site. In contrast, the unmanaged site had stronger relatedness in the juvenile stage than in adults, as is usually found in natural tree populations. Natural populations often show greater SGS in the early stages of regeneration, due to the higher spatial aggregation of trees (Rozas et al., 2009; Szwagrzyk and Czerwczak, 1993). This pattern has been reported in other species of *Pinus* (González-Martínez et al., 2002), in *Quercus* (Hampe et al., 2010), tropical trees (Hardesty et al., 2005; Ng et al., 2004) and other plant species (Yamagishi et al., 2007). Nevertheless, a few studies have found greater SGS in adult life stages, such as in *Jacaranda copaia* (Jones and

Hubbell, 2006), where it was attributed to very low recruitment and high mortality rates, or in the tropical tree *Dicorynia guianensis*, linked to overlapping of generations in the adult cohort (Latouche-Hallé et al. 2003). A subsequent study of the latter species found stronger SGS in saplings (Leclerc et al., 2015), suggesting that earlier observations were probably specific to the particular study cohort. Stronger SGS in adults than in late juveniles was also found for *Prunus africana* and it was attributed to a reduction in gene flow due to past logging (Berens et al., 2014). In our study, the most probable explanation seems to be the influence of changes in contemporary management. In the managed populations of Scots pine investigated here, high felling pressure at the time of establishment of the adult cohort, together with high browsing pressure, has suppressed regeneration for decades, which is also reflected in the absence of individuals estimated between 25 and 100 years old (Fig. 2). In the last 25 years, there has been a deliberate policy to encourage regeneration in the pine forest (Mason et al., 2004), with a consequent increase in forest density. This increment in forest density appears to have maintained diversity levels, increased gene flow and produced a more randomized distribution of genotypes in the new generation.

The observed reduction in juvenile *SGS* shows an erosion of the structure present in the adult cohort and contemporary recovery to natural dynamics, reflecting the high capacity of the species to recover after disturbance. Overall, *Sp* was higher in Glen Affric than in Abernethy, as for *SGS*. Although the spatial extent of *SGS* was higher

in adults at Glen Affric, *Sp* was slightly higher in the juvenile stage. This means more distant pairs of juveniles were less related than they would be by chance (juveniles showed a lack of relatedness among individuals at 50-80 m separation). The biological cause of this trend is not clear but, together with F_{ST} values that showed a small but significant difference among juveniles and adults, it may indicate introgression from populations not present in our sample.

4.5.3 Conclusions

In this study we investigated how historical and contemporary forest management have shaped patterns of genetic diversity and spatial distribution of genotypes of Scots pine. We provide evidence to show that although overall levels of genetic diversity in historically managed populations can be similar to unmanaged populations and as high as continental populations, spatial genetic structure can be considerably altered. Our results suggest that intense management practices that remove trees from the stand, such as felling, could alter fine-scale patterns of gene flow and increase genetic relatedness of individuals at fine scales with implications for inbreeding levels and, potentially, long-term adaptability. As a consequence, the extent of family clusters can be modified, as for instance in our study which increased up to 40 metres in managed sites. From a practical point of view, to ensure a broad sample of genetic variability, guidelines for seed collection should aim for minimum sampling distances between mother trees of at least 40m.

The reduction of SGS observed in juveniles following contemporary management to promote regeneration, indicates a high capacity of the species to recover after intense forest management. Here, we suggest that combining sustainable management with forest conservation practices that ensure larger effective population sizes is key to successfully maintaining genetic diversity in Scots pine. This capacity of the early stages of regeneration to capture gene flow might have implications for forest resilience and will be particularly important in the context of climate change (Alfaro et al., 2014; Fady et al., 2015; Hoffmann and Sgrò, 2011; Millar et al., 2007) under which selection pressures are expected to change.

Here we showed how investigating the spatial component of genetic diversity alongside tree demographic structure can help to detect both historical and contemporary effects of disturbances in tree populations. The effects of forest management were not reflected in overall levels of genetic diversity, but they were manifested in SGS, as has been seen in previous studies (Paffetti et al. 2012; Leclerc et al. 2015; Sjölund and Jump 2015). Therefore, incorporating a spatial component when evaluating the effects of forest management practices is highly recommended. The evaluation of successional change is also essential to properly assess genetic dynamics within populations and to adequately detect early responses to forest management practices.

4.6Acknowledgments

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4.8 Supplementary material

S4.1 Genetic diversity estimators for each locus, study site and life stage.

Locus	Life stage	Abernethy					Glen Affric					Unmanaged				
		N	*A*	A_R	H_E	F_{IS}	*N*	*A*	A_R	H_E	F_{IS}	*N*	*A*	A_R	H_E	F_{IS}
PtTX4001	Adult	181	11	9.28	0.8306	-0.028	165	9	7.59	0.7783	0.03	57	7	6.22	0.5951	-0.002
	Juvenile	170	12	9.32	0.8430	-0.06	131	11	8.79	0.8074	0.054	73	9	6.22	0.5073	0.028
PtTX4011	Adult	181	7	4.61	0.5920	0.099*	165	7	5.12	0.5423	0.213***	57	6	5.66	0.6717	0.204*
	Juvenile	170	6	4.73	0.6144	0.22***	131	6	5.05	0.6094	0.097	73	5	4.96	0.6922	0.3*
psy144	Adult	181	5	3.08	0.1166	-0.042	165	5	3.12	0.1380	-0.054	57	2	1.88	0.0517	-0.018
	Juvenile	170	5	2.88	0.0804	-0.024	131	5	3.2	0.1581	-0.067	73	3	2.39	0.1293	-0.06
psy117	Adult	181	8	6.32	0.7820	0.054	165	10	6.97	0.7907	-0.004	57	8	7.03	0.8224	-0.065
	Juvenile	170	8	5.98	0.7600	0.133**	131	8	6.56	0.7580	0.016	73	7	6.8	0.8247	-0.025
psy142	Adult	181	5	4.15	0.6466	0	165	6	5.22	0.6669	0.019	57	4	3.51	0.6479	-0.084
	Juvenile	170	6	4.34	0.6632	0.104*	131	6	5.07	0.6551	0.01	73	5	4.32	0.6411	-0.155*
psy12	Adult	181	3	2.17	0.3193	0.163*	165	3	2.18	0.2727	-0.096	57	2	2	0.3354	0.059
	Juvenile	170	3	2.17	0.3539	0.087	131	3	2.23	0.2386	0.393***	73	2	2	0.2314	-0.017
psy116	Adult	181	7	5.95	0.7862	-0.03	165	6	5.5	0.7736	0.011	57	6	5.5	0.7399	-0.092
	Juvenile	170	8	5.95	0.7720	0.063	131	7	5.42	0.7512	-0.024	73	6	5.87	0.7598	-0.01
psy157	Adult	181	5	4.23	0.3652	0.002	165	6	4.52	0.3483	-0.009	57	4	3.99	0.3892	-0.128
	Juvenile	170	5	4.19	0.3517	0.064	131	5	4.05	0.2984	-0.024	73	5	4.39	0.5168	-0.087
CTG4698	Adult	181	8	6.24	0.6044	0.019	165	8	5.17	0.5635	-0.043	57	5	5	0.6500	0.049
	Juvenile	170	6	5.34	0.6124	-0.034	131	6	5.27	0.5721	-0.068	73	5	4.64	0.6065	-0.016
SPAC7.14	Adult	181	29	19.08	0.9174	0.194***	165	26	18.6	0.9150	0.236***	57	22	17.95	0.9023	0.09*
	Juvenile	170	28	17.13	0.9093	0.179***	131	28	17.83	0.9072	0.21***	73	28	22.47	0.9513	0.097**
SPAC12.5	Adult	181	21	16.15	0.8989	-0.007	165	17	14.62	0.9058	0.098***	57	22	16.58	0.8475	0.048
	Juvenile	170	19	15.33	0.8956	0.054*	131	22	14.58	0.8814	0.005	73	19	15.85	0.8629	0.032
psy136	Adult	181	5	4.06	0.1877	0.438***	165	4	2.82	0.1166	0.216***	57	3	2.76	0.2607	-0.01
	Juvenile	170	5	3.23	0.1451	0.108	131	4	2.82	0.0897	-0.029	73	4	3.35	0.2578	-0.01

N, sample size; *A*, mean number of alleles per locus; A_R, rarefied allelic richness; H_E, expected heterozygosity; F_{IS}, inbreeding coefficient. Significant *P*-values are indicated as **P* < 0.05; ***P* < 0.01; ****P* < 0.001. *P*-values for F_{IS} are obtained after 10,000 permutations of gene copies within individuals of each stand.

S4.2 Pairwise F_{ST} values (below diagonal) and differentiation index *D* (Jost, 2008) (above diagonal) among study sites and life stages.

	ABE Adults	ABE Juveniles	GLA Adults	GLA Juveniles	UNM Adults	UNM Juveniles
ABE Adults	-	-0.00134	0.01367***	0.01694***	0.09089***	0.08407***
ABE Juveniles	-0.00085	-	0.01925***	0.01836***	0.09777***	0.09615***
GLA Adults	0.00531***	0.00504***	-	0.01223**	0.08486***	0.08469***
GLA Juveniles	0.00794***	0.00712***	0.00514***	-	0.09852***	0.09642***
UNM Adults	0.04973***	0.05174***	0.04434***	0.05228***	-	0.00843
UNM Juveniles	0.04923***	0.05132***	0.04586***	0.05382***	-0.00254	-

ABE refers to Abernethy, GLA refers to Glen Affric, UNM refers to the unmanaged site. Significant *P*-values are indicated as **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Chapter 5

Nearby Scots pine populations from contrasting climates show substantial population variability but consistent response to warming

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Intention to submit to Tree Physiology

Photos in Chapter 5 main page taken from P. González-Díaz.

5.1 Abstract

Natural tree populations consist on individuals that exhibit intraspecific adaptive variation at different geographic scales, as a result of the balance between gene flow and selection. The extent and magnitude of such variation will influence the adaptive capacity of trees under forthcoming changing environmental conditions. Scots pine in Scotland is the iconic species of the remnant Caledonian forest and appear in a relatively narrow geographic area with a steep East-West environmental gradient, increasing in rainfall and temperature. We hypothesised that western populations could be better adapted to warmer conditions and, as a consequence, could perform differently to predicted increased temperature. We performed an experiment under strictly-controlled conditions with a nested hierarchical design including region (west of east), population and families. We used two temperature scenarios, current temperature and increased temperature, and analysed germination and growth of above and below-ground traits. The largest amount of variation occurred at the family level. Nevertheless, regional and population differences were detected, where eastern populations invested more in roots and western populations had a greater number of stomata rows and thicker roots. In addition, increased temperature had strong effects on early growth similar in the two regions, with advanced germination, enhanced growth and biomass about 10 times, but it was not accompanied by shifts in biomass partitioning. Despite the intra-specific variation found in Scots pine populations, our results reveal consistent

effects of increased temperature on growth and germination, and that the level of cryptic genetic variation did not vary among regions.

5.2Introduction

Variation in selective pressures across the distribution of a species means fitness optima shift according to local environment. Over time populations should tend towards higher frequencies of genotypes near the local fitness optimum. However, gene flow acts in opposition to selection (Kawecki & Ebert 2004) and as a consequence populations show variation around the optimum, with a magnitude determined by the strength of selection. This intraspecific genetic variation, which determines adaptive potential, is a key part of plant functional ecology and as such has been the subject of increasing research particularly in the context of climate change (Hoffmann & Sgrò 2011, Alberto et al. 2013, Savolainen et al. 2013, Anderson 2016). In tree species, which are predicted to be highly exposed to rapid climate change due to long generation times, an understanding of adaptive variation will be crucial as we plan strategies to manage their resilience to the changes ahead.

During the last century global mean temperature has increased substantially and, if greenhouse gas emissions continue at current rates, it is predicted to continue to rise through the 21st Century (IPCC, 2013). Temperature plays an important role in seed germination, growth and survival (Arft et al. 1999, Chidumayo 2007, Walck et al. 2011), an effect that is particularly apparent at altitudinal limits (Harsch et al. 2009, Greenwood et al. 2015, Matías et al. 2017) or in cold areas where temperature-induced drought is not a limiting factor (Wilmking et al. 2004). Increases in global

temperature are therefore likely to have important consequences for species persistence and for forest population dynamics at different scales (Peñuelas & Boada 2003, van Mantgem et al. 2009, Matías & Jump 2015, Matías et al. 2016). Forecasting the impact of warming on individual tree species has been complex, as the local outcome of global changes is hard to predict. Furthermore, it is often assumed that species will respond homogeneously to warming but this discounts differences in the extent of local intraspecific adaptive potential, which may result in very distinct responses among populations. Studies at continental scales that have taken intraspecific variation into consideration have found that this aspect of diversity may be key to buffering the negative impacts of climate changes on the distribution of a species (Reich & Oleksyn 2008, Benito Garzón et al. 2011, Oney et al. 2013, Matías & Jump 2014, Valladares et al. 2014, Matías et al. 2017). However, adaptive responses to warming at local scales have received substantially less attention (but see Wilmking et al. 2004; Benito Garzón et al. 2011).

Adaptive divergence has been found among tree populations at relatively small geographic scales, most probably due to spatial heterogeneity in local selection pressure (Salmela et al. 2013, Donnelly et al. 2016). However, much genetic diversity is of low adaptive significance in the home environment or involves genes that may only become selectively important in novel environments and is therefore 'cryptic' until exposed to new conditions. The release of this cryptic variation by a shift in environment such as climate change, can provide additional variation on which

selection can act and may facilitate adaptive tracking of the environment by the population. Therefore, there is a need to understand both the extent of individual population adaptive variation and how variation in adaptive traits might interact with warming in order to improve our ability to predict the response of particular tree populations under global warming conditions.

In plants, natural selection acts very strongly on the seedling life stage, and is when the greatest proportion of a generational cohort is lost (Schupp 1995). Their size and fragility makes them particularly susceptible to disturbances, such as competition, browsing, extreme climatic events, and insect or disease infestation, compared to adult plants (Vizcaíno-Palomar et al. 2014), and they may respond more rapidly to environmental changes than adult trees (Lloret et al. 2009). Consequently, consideration of germination and establishment, which are key fitness traits, will be critical to understanding the response of a tree to novel environments and to identify the abiotic drivers to which populations are locally adapted (Alberto et al. 2013). Assessment of the differential contribution of above and below ground traits can provide a useful way to characterize plant growth and resource use strategies, and has been shown to vary considerably within species (Hajek et al. 2013, Donnelly et al. 2016). Above ground traits, such as leaf morphology and stomatal abundance can provide useful information as leaves are key organs for photosynthesis, carbon assimilation and exchange of gas and water with the atmosphere, and the stomatal density influences rates of gas exchange and water loss (Donnelly et al. 2016).

Similarly, below-ground traits, which show large variation among species (Comas & Eissenstat 2009), can also be informative regarding intraspecific responses, as temperature has an important role in regulating belowground physiology and soil temperature can influence root growth, cell elongation, root length and diameter extension, initiation of new lateral roots and root branching patterns (Pregitzer et al. 2000).

Scots pine (*Pinus sylvestris* L.) is one of the most widely distributed tree species in the world and at a continental scale shows strong patterns of intraspecific adaptive variation (Andersson & Fedorkov 2004, Matías & Jump 2014, Matías et al. 2014). In Scotland, Scots pine represents the north-western limit of the species distribution and a globally important peripheral population, which might contain unique genetic resources. Several millennia of isolation with respect to the mainland populations have not led to the loss of genetic diversity in the Scottish populations, and they usually show high levels of neutral (Sinclair et al. 1998, Wachowiak et al. 2011), and adaptive genetic variation (Perks & Mckay 1997, Salmela et al. 2011, 2013, Perry et al. 2016, Donnelly et al. 2016) among and within populations. The remnant native pine forest in Scotland experience a generally temperate and oceanic climate, but are scattered over heterogeneous sites, which vary in aspect and altitude, and increase in mean temperature and rainfall patterns from East to West, with markedly different climatic conditions (namely temperature, precipitation and growing season length) at the extremes (Salmela et al. 2010). Therefore, the population constitutes a valuable

system in which to study underlying processes of intraspecific adaptive variation and to test whether populations are all likely to respond equally to novel forecasted environmental conditions.

Controlled environmental studies designed to mimic environmental conditions and/or predictions under climate change constitute an appropriate way to evaluate intraspecific variation in trait responses in young trees and to disentangle the underlying processes (environmental vs. genetic) of such responses to current and novel pressures (e.g. warming). This is possible, because by using common environmental conditions, environment is controlled which allow to assign any differences in the phenotype to the genotype alone. The main objective of this study was to characterize the intraspecific adaptive variation in Scots pine early growth traits (seed mass, seedling emergence, biomass accumulation and key above & below traits) among and within populations, relate this to spatial variation in home site environment, and estimate the likely response of those populations to projected climate change. Specifically, we sought to address the following questions: (1) do early growth traits under current temperatures differ in populations from western compared to eastern Scotland – is adaptive genetic divergence evident? (2) does the extent of variation in response to warming differ in populations from the west compared to the east of Scotland – does the level of cryptic genetic variation vary among populations? if so, does this correlate with historical exposure to stress (e.g. do populations from colder conditions show greater variation?). We hypothesized

that (1) early growth traits will differ in populations from the west compared to those in the east as a consequence of the steep longitudinal climatic gradient under current conditions, and (2) response to temperature warming on early growth traits will be stronger in populations from the eastern range, as cold temperatures are currently more limiting in populations from those areas, which also will show a greater amount of cryptic genetic variation.

5.3 Material and methods

5.3.1 Study populations and seed collection

Cones were collected in the winter of 2015 from mother trees growing in four natural populations representing two of the most westerly and two of the most easterly populations in Scotland (Table 5.1). With the objective of capturing maximum variation within each population, mature cones were collected from mother trees located in transects at three altitudinal levels, covering the whole altitudinal range of each population (Table 5.1). Four families were sampled in each altitudinal level (12 families per population in total), from mother trees separated by at least 40 metres, as family clusters of Scots pine can occur at distances of less than this in Scottish pinewoods (González-Díaz et al. 2017). The term family here will refer to a group of individuals who share a maternal parent (half sib families). Altitude was subsequently found not to explain significant variation in the studied traits, and is not dealt with further here. Therefore, to summarize, the sampling design was: 2

regions x 2 populations per region x 12 families per population, resulting in 4 populations and 48 families sampled in total.

Table 5.1 Location of study sites and altitudinal ranges where the mother trees were sampled, as well as mean temperature and precipitation in each location.

Population	Code	Latitude	Longitude	Altitudinal Range	Altitude range of transect per site			Mean annual Temp	Mean annual Rainfall
					Low	Medium	High		
Glen Tanar	GT(E)	57.02	2.86	276-418	276-278	323-350	404-418	7.40	780
Glen Derry	GD(E)	57.03	3.58	438-525	438-464	466-493	508-525	6.80	932
Benn Eighe	BE(W)	57.63	5.40	9-222	9-20	41-140	196-222	8.53	2282
Shieldaig	SH(W)	57.50	5.63	6-242	6-49	111-144	230-242	8.40	1467

Seeds were extracted after cones were oven dried at 45°C for 3-4 days. Cone size was classified as big, medium or small and seeds were pooled within a maternal progeny. Number of viable and non-viable seeds was visually evaluated and recorded, using full seeds as a proxy of viability in contrast to empty seeds. Viability was defined as the proportion of viable seeds present in the total set of seeds (viable plus non-viable). Viable seeds were weighed to estimate mean seed biomass per family within each population.

5.3.2 Experimental design

Ten replicates of each of the 48 families were sown on June 2015 at the Controlled Environmental Facilities of the University of Stirling (UK) in individual pots, and the experiment was conducted over the course of one growing season. Tubular pots (6.5 cm diameter, 45 cm height), were filled with a 2:1 mixture of peat and river sand. A bottom layer of gravel was used to improve drainage. Before sowing each pot was irrigated with 200 ml of soil microbial inoculum resulted from the maceration of

roots and soil beneath adult Scots pine trees growing at the University of Stirling, in central Scotland, to favour formation of mycorrhizas (Matías & Jump 2014). Three seeds were surface sown in each pot, thinning to one when multiple seedlings emerged, and retaining the first one to emerge. In total 480 pots were randomized in blocks inside four Snijders Scientific MC1750E (Tilburg, Netherlands) controlled environment chambers (inner space 1.8 m length × 0.75 m wide × 1.2 m high), resulting in 120 pots in each chamber. Since we applied two temperature treatments, two chambers were used for each of the treatments, each containing 5 replicates of the 48 families (240 pots in each treatment).

The experimental design was constructed with a nested structure to examine families within populations. Two temperature treatments were applied during the experiment: half of the samples (48 families belonging to the four populations with 5 individual replicates) were grown in current temperature (CT) and the other half in predicted future temperature (FT). Temperature for the CT treatment was set up by calculating the mean daily and night values for each month of the growing season using weather stations located near to the sampled populations for the period 1981-2010: Kinlochewe (57.613N, -5.308W, 25 m.a.s.l.) and Aultbea (57.859N, -5.636W, 11 m.a.s.l.) nearest to the western populations, and Aboyne (57.077N, -2.836W, 140 m.a.s.l.) and Braemar (57.006N, -3.397W, 339 m.a.s.l.) nearest to the eastern populations, available at http://metoffice.gov.uk. The FT treatment was set up with an increase of 5°C over that of CT, at both day and night temperatures (Table 5.2),

which is the likely increase in mean temperature under the UKCP09 high emission scenario by 2080 at the 50% probability level (http://ukclimateprojections.metoffice.gov.uk/). This high emission scenario is based on the SRES A1F1 emission scenario (IPCC, 2013). Day and night temperatures were set up with a constant duration of 16 and 8 hours respectively (experimental temperature values are summarised at Table 5.2). Light intensity was fixed for 16 h with a photosynthetic photon flux density of 210 mol m^2 s^{-1}, rising progressively at dawn and decreasing at dusk for 1 h, which is the value representative for forest understory (Valladares et al., 2004). The appropriate watering was estimated by calculating the mean rainfall value for each weather station during the growing season (May to September), for the period 1981-2010, resulting in monthly precipitation levels of 86.225 l/m^2. Watering was applied twice per week, assuming therefore that water availability was not a limitation in our study. Air relative humidity and CO_2 concentration were kept constant at 70% and 891 mg m^{-3} (460 ppm) respectively. To minimise any possible chamber effect, all pots were rotated between the different chambers, spending at least one month in each chamber after programming for the appropriate treatment conditions, whilst also randomising block position within chambers. However, block composition was kept constant during all the experiment. Soil moisture was measured every ten days during the experiment in all pots over the surface 5 cm by the time-domain reflectometry method (SM300; Delta-T devices, Cambridge, UK), and values were recorded two

days after irrigation. Monitoring of emergence was carried out every two days until the last seedling emerged.

Table 5.2 Temperature (day/night) during the experiment for the two treatments: Current Temperature (CT) and Future Temperature (FT).

Week	Equivalent	CT		FT	
		Day Temp. (16h.)	Night Temp. (8h.)	Day Temp. (16h.)	Night Temp. (8h.)
1-4	May	14° C	6° C	19° C	11° C
5-8	June	16° C	8° C	21° C	13° C
9-13	July	18° C	10° C	23° C	15° C
14-17	August	17° C	9° C	22° C	14° C
18-22	September	15° C	7° C	20° C	12° C

On 26th November 2015 after 22 weeks in the controlled environment chambers plants were harvested and cut at the root collar to divide each plant into above and belowground parts. Roots were washed gently but thoroughly taking care to keep loss of root tissue to a minimum, and measurements of maximum root and shoot length and fresh biomass were taken. Five needles were collected from each seedling, two from the upper part, two from the lower and one from the middle of the plant. The number of stomatal rows at midway along the needle on its adaxial (upper) surface was counted with the aid of a stereo microscope Leica light microscope (x10 objective). Stomatal rows were used as a proxy of stomatal densities. After measurements, needles were scanned using a scanner at 300 d.p.i. and placed together with the rest of its seedling. Data measurement of scanned needle dimensions was done using ImageJ software, v.1.36b. (Abramoff et al., 2004). Clean fresh roots were placed flat on a sheet of paper and also scanned. The obtained images were edited manually with Adobe Photoshop® in order to remove any

shadows that would cause spurious measurements to be recorded. Data acquisition for structural and morphological analysis of scanned roots was done using WinRhizo (Regent Instruments Inc., Quebec, Canada, 2000), obtaining the following morphological parameters of roots, which are averaged for every root (e.g. considering all main and fine roots): total root area, total root length, root diameter, root volume, number of root tips and forks. Dry biomass (g) was recorded after both roots and shoots were dried in the oven for a minimum of 3 days at 45°C.

In total, nineteen early growth traits subject to differing natural selection were considered for the following statistical analysis (Supplementary S5.1) of which two refer to the seed stage, mean viable seed weight (SEW) (mg) and seed viability (SEV) (%). Seedling emergence (EME) was recorded as soon as the seeds were observed at the substrate surface within pots, and survival (SUR) (%) was checked every two days and recorded over the duration of the experiment. All the remaining traits were recorded at the end of the experiment: Total dry biomass (TDB), which is the sum of shoot dry and root dry biomass (g); plant height (SHL) (mm); maximum root depth (ROL) (mm); root mass fraction (RMF) which is the ratio of root dry biomass to total dry biomass. It is worth noting that RMF was used instead root : shoot (R:S) ratio, commonly used in other studies, as RMF values lie between 0 and 1, which makes comparisons between plants of different sizes easier compared to R:S which is unconstrained and can vary from a tiny to a very large number (Pérez-Harguindeguy et al. 2013). Other below-ground traits: total root length (TRL), which

represents the sum of the lengths of all the fine roots (cm); average root diameter (AVD) (mm); root volume (ROV) (cm^3); number of tips (TIP); number of forks (FOR); branching intensity (BRI), which is the ratio of the number of tips to the total root length (cm^{-1}); specific root area (SRA), which is the ratio of surface area to root dry biomass ($m^2\ g^{-1}$) and the specific root length (SRL), which is the ratio of total root length to the root dry biomass ($m\ g^{-1}$). Finally, above-ground traits, needle length (NEL) (mm), needle width at its midpoint (NEW) (mm) and the mean number of stomatal rows (STO) were measured in five needles per individual. Mean values are given ± Standard deviation.

5.3.3 Data analysis

5.3.3.1 Seeds and seedling emergence

A nested analysis of variance (ANOVA) was used to test differences in seed biomass and viability among and within populations. To analyse differences in emergence, only the date of emergence of the first of the three seeds shown per pot was considered, and the emergence ratio on each experimental day was calculated. A cumulative binary logistic regression was used to evaluate the effect of temperature, population and experimental day of emergence in the emergence ratio with the glm function, using a binomial error distribution. Experimental day, temperature, population and the interaction between the three factors were considered as the

predictor variables. Survival was estimated as the proportion of emerged seedlings that survived to the end of the experiment.

5.3.3.2 Intraspecific variation of early growth traits and response to temperature

Mixed effect models were used to analyse the genetic variance components of the response of early growth traits and to test the effect of temperature with the statistical package "lme4". To identify the best-supported model we constructed all possible combinations of alternative models, from the maximal model considering both the main effects and the pair-wise interactions between the fixed effects. Models were fitted by *Maximum Likelihood* (ML) and model selection was performed using backward stepwise selection to minimize the *corrected Akaike Information Criterion* (AICc). The selected model included temperature and region as fixed factors (and the interaction of both for few traits) and population, family and block as a random factor, and it was fitted with a *Restricted Maximum Likelihood* (REML) algorithm to obtain parameter estimates.

$$Trait = \mu + Temperature_i + Region_j + Population_k + Family_{l(k)} + Block_m + \varepsilon_{ijklm} \qquad (1)$$

This model (1) was applied for two data-sets: (i) all data containing the source of variation "temperature", and (ii) data separately by temperature treatment, removing the source of variation "temperature" from the Eq. (1). Residuals were

tested for normality and homoscedasticity, and log transformation of the variables was applied when necessary.

(i) The model containing all data, which included the temperature treatment, was analysed in order to detect the variance explained for each of the factors. To estimate the variance of the trait of interest associated with a specific fixed effect, the *k*-th, we used the Eq. 2.

$$\sigma^2_{fk} = \frac{var(\beta_k x_{ik})}{\sum_j var(\beta_k x_{ij})} \sigma^2_f \quad (2)$$

Where x_{ij} is the explanatory variable data for variables $j = 1, \ldots, p$, and β_k is the estimated fixed effect.

(ii) The models for the individual temperature treatments were analysed in order to calculate the variance explained for each of the factors and to estimate additive genetic variation, heritability and evolvability of the trait of interest in each treatment. Narrow-sense heritability (h^2), which corresponds to the proportion of total phenotypic variation attributed to additive genetic variation, proportional to the rate of short-term responses to selection (Bolnick et al. 2011), were estimated as indicated in Eq. 3

$$h^2 = \frac{V_A}{V_P} = \frac{4\, V_{fam}}{V_{fam} + V_{block} + V_{res}} \quad (3)$$

Where V_A is the additive genetic variance and V_P is the phenotypic variance; V_{fam}, V_{block}, V_{res}.

Standard errors (*SE*) for heritabilities (h^2) were estimated as suggested by (Visscher 1998), indicated in Eq. 4.

$$SE_{h^2} = 4\sqrt{\frac{2(1-\frac{h^2}{4})^2\,[1+(s-1)\frac{h^2}{4}]^2}{s(s-1)(f-1)}} \quad (4)$$

Where s is the number of individuals per family, and f is the number of families.

The genetic coefficient of variation CV_A was estimated according to (Houle 1992) following Eq. 5, and is a standardised measure of variation normalised by the trait mean. It provides a measure of the evolvability of a trait.

$$CV_A = \frac{\sqrt{V_A}}{\mu_{\text{Trait}}} \times 100 \quad (5)$$

Where μ_{Trait} is the mean of the trait of interest.

Differences among regions for each temperature treatment were evaluated by comparing pairs of models by AIC_C for each of the studied traits. The model with the same formula as equation (1) without the temperature factor, with region as a fixed effect, was compared to a null model which did not include the region effect.

To detect differences in soil moisture among the different treatments and regions (or populations), repeated measures ANOVA was used. All statistical analyses were carried out in R (http://www.R-project.org/) in order to assess the level of intraspecific variability at different levels (region or population and family) and the effect of the predicted increase of temperature.

5.4 Results

5.4.1 Seeds and seedling emergence

Viability of seeds differed significantly among regions (F= 1.56, $d.f.$=1, p=0.04) and populations (F= 4.00, $d.f.$=3, p=0.01) which ranged from the lowest viability at BE(W) (61.73% ± 18.79) and SH(W) (62.08% ± 11.55) followed by GD(E) (65.35% ± 5.36), and the highest seed viability was at GT(E) (70.62% ± 9.47). Those differences were not accompanied by differences in seed biomass among regions nor among populations, whose mean values were similar for SH(W) (5.99 mg ± 0.95), BE(W) (5.66 mg ± 1.14), GD(E) (5.25 mg ± 1.24) and GT(E) (5.88 mg ± 1.06). In addition, and as expected, seed biomass differed among cone sizes (F=5.98, $d.f.$=2, p=0.006) (i.e. larger cones had heavier seeds).

A total of 478 seedlings emerged during the experiment (99.6%), considering only the seedling that was kept from the initial three, with the first day of germination nine days after sowing. Temperature had a strong effect in controlling the emergence ratio (z=-3.84, p=0.00123), with the warmer temperature advancing the date of emergence by approximately 10 days (mean time to emergence, CT= 26.23 ±8.39, FT=15.32 ± 4.08) (Table 5.3, Fig. 5.1). Day of emergence had a significant effect on the emergence ratio of a seedling in a population (z=32.51, p<0.001), indicating that the rank order of emergence for populations changed over time. Day of emergence had a significant interaction with population, indicating that the change over time of the emergence rate differed among populations, and a significant interaction with

treatment, indicating that emergence of the first seedling occurred earlier under FT but subsequent seedlings also emerged faster than under CT. Furthermore, significant differences among populations were detected, which were greater among the both eastern populations GT(E) and DG(E) than among western and eastern populations. Treatment x population interaction was only significant for BE(W). Furthermore, triple interactions between population, day and treatment were also observed (Table 5.3, Fig. 5.1). Survival was 100% on both treatments during the experiment.

Figure 5.1 Predicted cumulative emergence ratio over time (experimental day) across temperature treatments (Current Temperature, C and Future Temperature, F) and population (GD(E) GT(E), BE(W), SH(W)) using a binomial error distribution. Grey dots represent observed data.

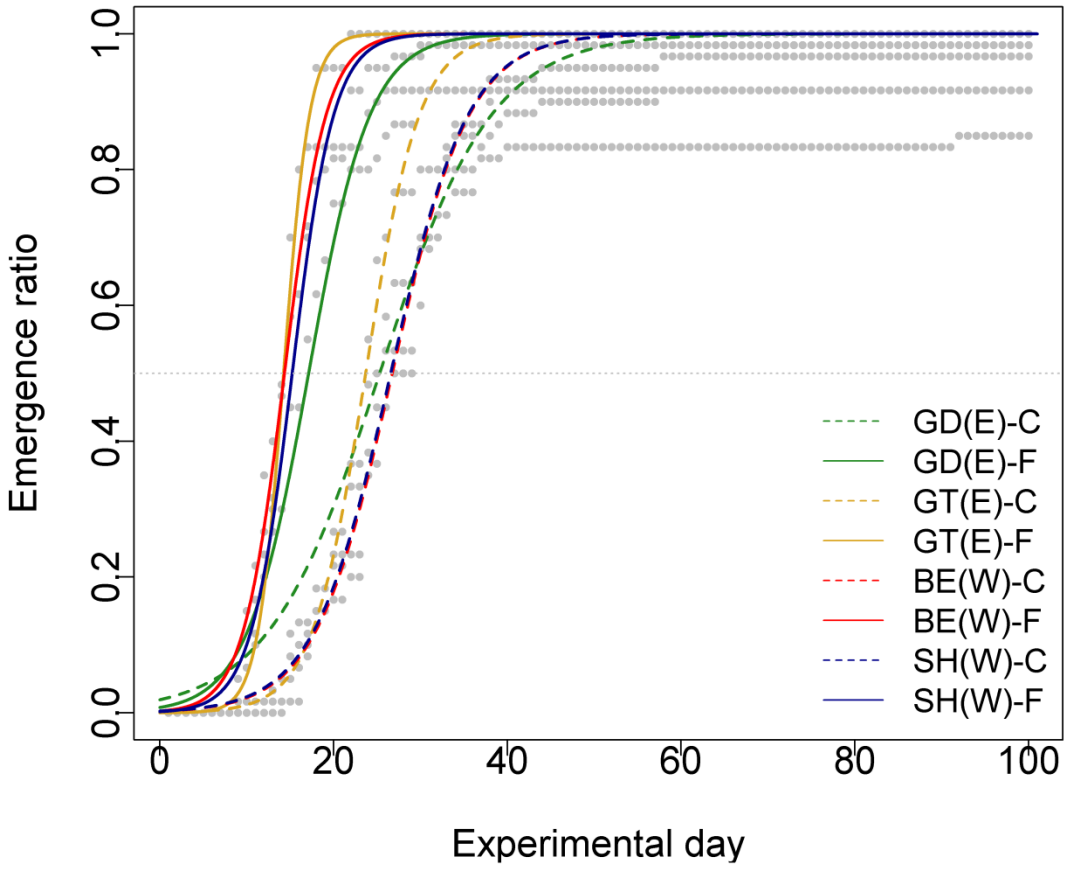


Table 5.3: Parameters of the Logistic Regression of cumulative emergence of Scots pine

Variables	Estimate	SE	z value	Pr(>|z|)
(Intercept)	-3.92	0.14	-27.11	<0.0001***
DAY	0.16	0.00	30.14	<0.0001***
TreatmentF	-0.91	0.25	-3.59	0.000327**
PopulationET	-3.72	0.36	-10.21	<0.0001***
PopulationBE(W)	-2.10	0.26	-7.95	<0.0001***
PopulationSH(W)	-2.03	0.26	-7.60	<0.0001***
DAY:TreatmentF	0.12	0.01	10.24	<0.0001***
DAY:PopulationET	0.16	0.01	11.43	<0.0001***
DAY:PopulationBE(W)	0.07	0.01	7.44	<0.0001***
DAY:PopulationSH(W)	0.07	0.01	7.21	<0.0001***
TreatmentF:PopulationET	-0.98	0.70	-1.38	ns
TreatmentF:PopulationBE(W)	0.96	0.46	2.1	0.035705*
TreatmentF:PopulationSH(W)	0.49	0.46	1.07	ns
DAY:TreatmentF:PopulationET	0.22	0.04	5.07	<0.0001***
DAY:TreatmentF:PopulationBE(W)	0.06	0.02	2.55	0.010775*
DAY:TreatmentF:PopulationSH(W)	0.07	0.02	2.71	0.006769**

Significant *P*-values are indicated as *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, non significant

5.4.2 Intraspecific variation of early growth traits

All traits showed substantial intraspecific adaptive variation, and the largest proportion of genetic variation was partitioned within the populations (among families) rather than among regions or populations for most traits. Family variance was relatively large for most traits (V_{FAM}, CT: 0-17.43 %) (Table 5.4), and those values were reflected in the high estimates of narrow-sense heritability (h^2, CT: 0-0.77). Also the estimates of evolvability were relatively large (CV_A, CT: 0-48.31). Although variance attributable to region was smaller than that for family in the majority of cases, it was nevertheless significant for different traits (V_{REG}, CT: 1.61-4.99 %), as was indicated by the adequate support for the inclusion of region as a fixed effect for those traits in CT (ΔAIC, CT=1.68-5.15) (Table 5.4). Furthermore, differences among

populations were also present in most of the traits (V_{POP}, CT: 0.61-9.01 %) (but see RMF, BRI, SRA, SRL).

Table 5.4 Genetic components for the current and future temperature model: the proportion of variance attributable to population, family within population, block and residual which refer to the individual; heritabilities (h^2) with their associate error, and evolvabilities of the traits (CV_A). Model subsets were generated and compared by AICc to assess the importance of Population. The best models were determined by AICc, and the presence (+) or absence (-) of fixed effects are indicated for each of the traits listed. ΔAICc values represent the difference in AICc between the null model and the best (a value of zero indicates that the null model was the most parsimonious).

Treatment	Trait	Trait code	Region	df	ΔAIC	AIC	V_{REG} (%)	V_{POP} (%)	V_{FAM} (%)	V_{BLOCK} (%)	V_{RES} (%)	h^2 (SE)	CV_A
CT	Total Dry Biomass	TDB	-	5	0.00	344.62	-	9.01	17.43	3.61	69.96	0.77 (0.26)	15.44
	Shoot Height	SHL	-	5	0.00	447.46	-	4.73	0.88	13. 67	80.71	0.04 (0.19)	4.52
	Root Depth	ROL	-	5	0.00	185.33	-	4.13	5.99	0.62	89.25	0.25 (0.21)	3.08
	Root Mass Fraction	RMR	+	6	5.15	-611.78	4.99	0.00	0.00	1.83	93.18	0.00	0.00
	Total Root Length	TRL	-	5	0.00	383.94	-	6.05	9.48	4.62	79.84	0.40 (0.18)	8.59
	Average Diameter	AVD	+	6	1.71	-651.39	4.46	4.04	4.05	25.92	59.86	0.18 (0.18)	6.89
	Root Volume	ROV	-	5	0.00	462.09	-	7.28	9.10	8.27	75.34	0.39 (0.18)	16.82
	Tips	TIP	-	5	0.00	449.03	-	5.77	9.25	4.94	79.61	0.39 (0.18)	7.84
	Forks	FOR	-	5	0.00	442.55	-	5.61	7.22	20.41	66.75	0.31 (0.18)	6.96
	Branching intensity	BRI	-	5	0.00	416.62	-	-	9.10	0.62	90.29	0.36 (0.18)	12.35
	Specific Root Area	SRA	+	6	1.68	-1250.11	1.61	-	9.11	10.62	78.65	0.37 (0.18)	23.21
	Specific Root Length	SRL	+	6	4.38	1640.12	5.61	-	10.04	9.62	74.72	0.43 (0.18)	22.08
	Needle Length	NEL	-	5	0.00	-121.97	-	5.73	11.58	4.94	77.75	0.49 (0.18)	5.93
	Needle Width	NEW	+	6	2.30	-1117.63	2.19	0.61	11.4	3.27	82.49	0.47 (0.18)	48.31
	Stomatal Rows	STO	+	6	3.28	-116.60	6.55	1.97	16.03	0.96	74.27	0.70 (0.26)	11.62
FT	Total Dry Biomass	TDB	+	6	0.35	704.23	1.95	0.00	12.79	7.94	77.32	0.52 (0.24)	66.58
	Shoot Height	SHL	-	5	0.00	568.22	-	2.90	5.95	3.77	87.37	0.25 (0.21)	9.90
	Root Depth	ROL	-	5	0.00	172.08	-	2.44	0	3.74	93.81	0.00	0
	Root MassFraction	RMR	-	5	0.00	-534.06	-	1.58	6.59	4.45	87.39	0.27 (0.22)	9.24
	Total Root Length	TRL	-	5	0.00	658.07	-	1.60	13.55	8.04	75.66	0.55 (0.18)	12.78
	Average Diameter	AVD	+	6	2.69	-573.97	5.75	0.91	0	6.20	87.14	0.00	0
	Root Volume	ROV	+	6	0.10	743.48	2.27	0.93	10.14	7.05	79.62	0.42 (0.23)	14.52
	Tips	TIP	-	5	0.00	605.86	-	1.04	14.20	8.26	76.50	0.57 (0.25)	10.09
	Forks	FOR	-	5	0.00	678.20	-	1.64	13.33	14.78	70.25	0.53 (0.24)	10.93
	Branching intensity	BRI	+	6	2.90	418.89	3.56	0	1.14	1.21	94.08	0.07 (0.19)	5.91

Specific Root Area	SRA	-	5	0.00	-1430.23	-	0.62	0.00	2.95	96.42	0.00	0
Specific Root Length	SRL	+	6	1.47	1621.58	4.10	1.27	0.52	3.99	90.13	0.02 (0.19)	3.72
Needle Length	NEL	-	5	0.00	888.49	-	1.81	19.74	2.27	76.18	0.80 (0.26)	9.91
Needle Width	NEW	-	5	0.00	-161.09	-	0.37	12.94	3.45	83.24	0.52 (0.24)	66.08
Stomatal Rows	STO	+	6	2.02	-51.92	2.88	-	15.46	1.69	79.96	0.68 (0.25)	11.71

Data from table 6 include variance components for region (V_{REG}), population (V_{POP}), family (V_{FAM}), block (V_{BLOCK}) and residual (V_{RES}).

Regional differences were evident for some early growth traits, where the western populations displayed smaller biomass allocated to roots (RMF) compared to the eastern ones in CT (Fig 5.2b). Remarkable differences were evident among regions in other root traits, with western populations having thicker roots (AVD) (Fig. 5.3a), shorter specific root length (SRL) (Fig. 5.3b) and lower specific root area (SRA). The number of root tips was greater in the eastern population GT(E) for CT but did not show any trend in the W-E range. Branching intensity was higher in SH(W) followed by BE(W), compared with eastern populations, however differences among regions were only significant under the temperature treatment FT (Fig. 5.3c). Regarding above ground traits, western populations had significantly greater number of stomatal rows (Fig. 5.4a) and thicker needles in CT (Fig. 5.4b). Needle length changed among populations, with SH(W) and GT(E) having the longest needles in CT, whilst GD(E) had the shortest ones, but no regional differences were present. Population differences were also evident for some early growth traits, where the western population SH(W) displayed greater biomass (TDB) and longer roots (ROL) and shoots (SHL) at CT whereas both eastern populations GT(E) and GD(E) had the lowest values (Fig. 5.2a and 5.3a), however, no regional differences were observed in those traits.

Figure 5.2 Total dry biomass (a) and root mass fraction RMF (b) of Scots pine seedlings from the four studied populations across the temperature treatment (Current T, Future T). Refer to table 1 for population code key. Error bars represent± SE.

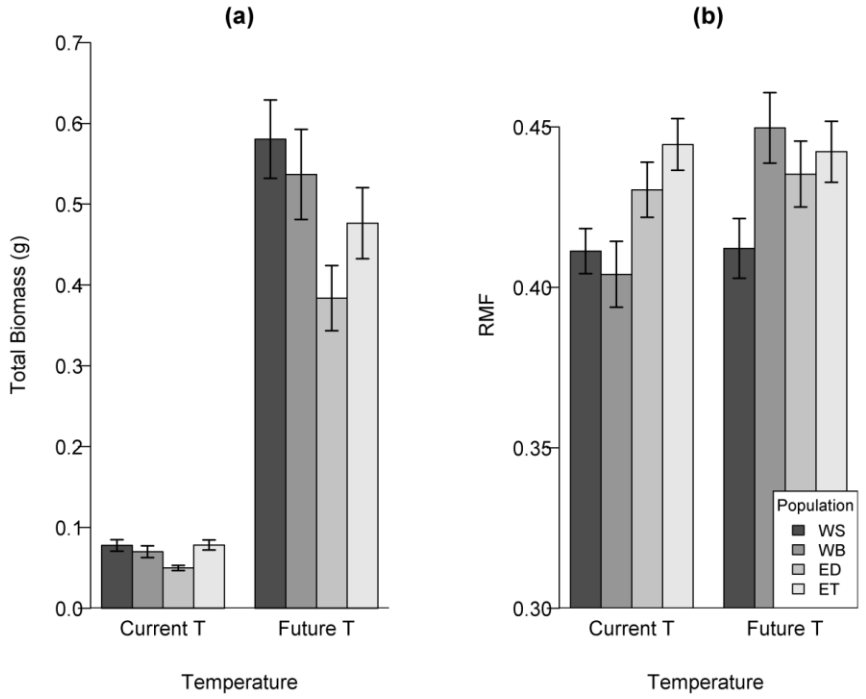


Figure 5.3 Below-ground traits: root average diameter (a), Specific Root Length SRL (b) and branching intensity (c) of Scots pine seedlings from the four studied populations across the temperature treatment (Current T, Future T). Refer to table 1 for population code key. Error bars represent ± SE.

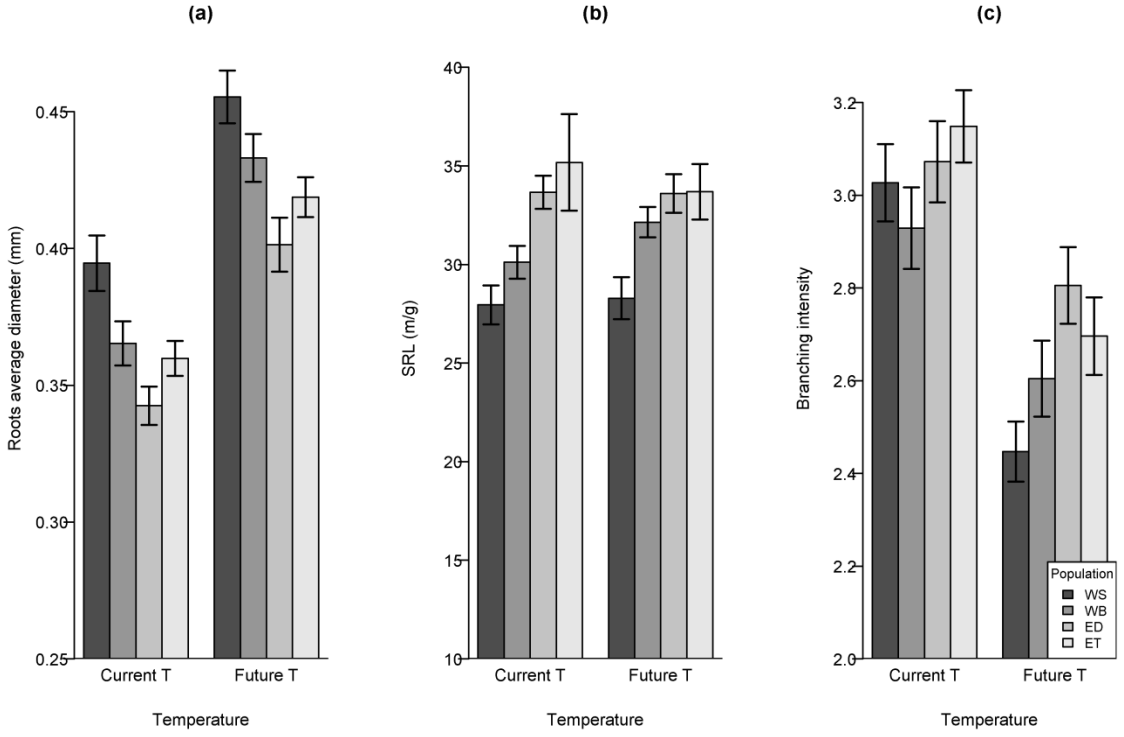
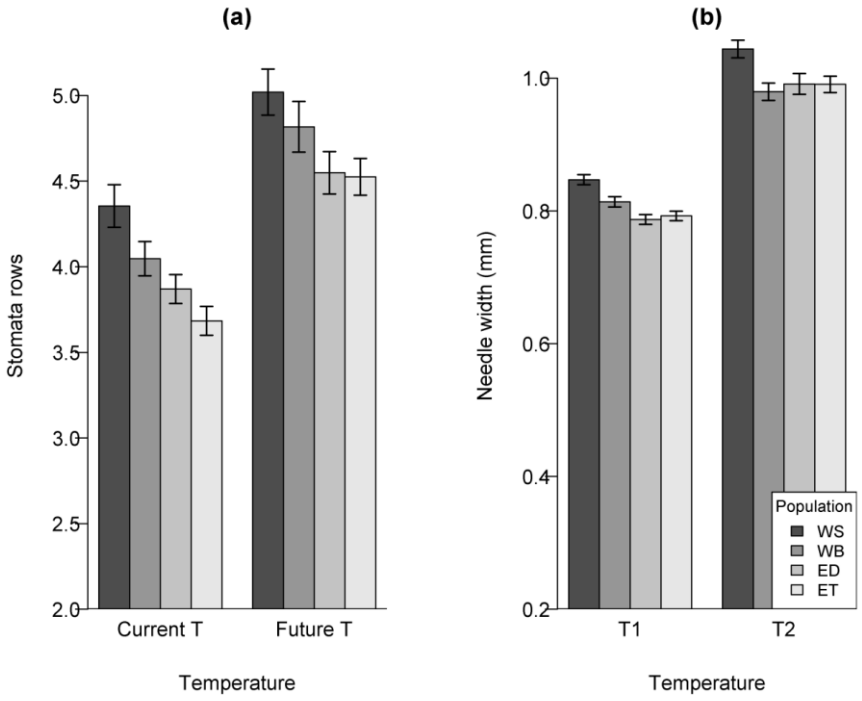


Figure 5.4: Above-ground traits: number of stomata rows (a) and mean needle length (b) of Scots pine seedlings from the four studied populations across the temperature treatment (Current T, Future T). Refer to table 1 for population code key. Error bars represent ± SE.



5.4.3 Response to temperature of early growth traits

Soil moisture availability was different among treatments (F=101.14, $d.f.$=1, p<0.0001), due to the different temperature levels imposed during the experiment, however no significant differences were observed among regions or populations within treatments (F=1.968, $d.f.$=3, p=0.1179).

Overall, no temperature x region interactions were found for the majority of the traits assessed in the study, which means that all populations responded consistently under current and increased temperature (but see TRL and FOR in Table 5.5). Temperate had a strong effect on most of the measured traits, and explained up to 49.33% of the variance (Table 5.5). The temperature treatment (FT) resulted in

significantly greater total biomass (TDB, CT: 0.069 g ± 0.049, FT: 0.49 g ± 0.37) (Fig. 2a), taller shoots (SHL, CT: 17.16 mm ± 9.35, FT: 64.15 mm ± 34.85) and deeper roots (ROL, CT: 300.26 mm ± 91.43, FT: 435.57 mm ± 99.55) (we did not observe root growth limitation due to pot height). However, with the exception of population BE(W) which invested relatively more into roots in the FT treatment, there was no or little change in biomass allocation (RMF, CT: 0.42 ± 0.067, FT: 0.43 ± 0.078), (Fig. 2b). Most of the below ground traits were highly influenced by the warmer temperature treatment, which resulted in longer (e.g. considering the total length) (TRL, CT: 86.63 cm ± 60.04, FT: 608.36 cm ± 420.23), thicker (AVD, CT: 0.36 mm ± 0.061, FT: 0.43 mm ± 0.07) (Fig.3a), and more voluminous roots (ROV, CT: 0.1 cm^3 ± 0.071, FT: 0.99 cm^3 ± 0.83), with more forks (FOR, CT: 370.54 ± 305.88, FT: 2976.11 ± 2434.58) and more root tips (TIP, CT: 267.24 ± 186.80, FT: 1449.04 ± 905.84). However, the warmer temperature reduced the branching intensity of roots (BRI, CT: 3.04 cm^{-1} ± 0.622, FT: 2.63 cm^{-1} ± 0.60) (Fig. 3c), and had little or no effect on SRA, (SRA, CT: 0.04 mg^2 g^{-1} ± 0.013, FT: 0.04 mg^2 g^{-1} ± 0.01) and SRL (SRL, CT: 31.79 mg g^{-1} ± 10.89, FT: 31.89 mg g^{-1} ± 8.39) (Fig. 3b) respectively. Warmer temperature also had a strong effect on the above ground traits, resulting in greater numbers of stomatal rows (STO, CT: 3.99 ± 0.79, FT: 4.73 ± 1.01) (Fig. 4a) and longer (NEL, CT: 17.9 mm ± 4.57, FT: 33.37 mm ± 11.29) and thicker needles (NEW, CT: 0.81 mm ± 0.13, FT: 1.001 mm ± 0.23) (Fig. 4b).

Predicted warming (FT) diminished the differences among populations, which is reflected in the reduction of the V_{POP} (CT: 0.61-9.01 %, FT: 0-2.90 %) for all traits

except for RMF, SRL and SRA (see Table 5.4). However, the west-east regional differences (V_{REG}), were increased under the FT treatment for some traits (TDB, AVD, ROV and BRI), while they were decreased for others (RMF, SRA, SRL and TWN) (See Table 5.4). Neither narrow sense heritability showed consistency in the direction in which it shifted in response to the warmer temperature treatment (Table 5.4). However, for most of the traits not related with roots C_{VA} was increased (Figure 5.5) under warmer temperatures.

Figure 5.5 Plasticity to change of the coefficient of genetic variation (C_{VA}) of Scots pine seedlings across the temperature treatment (Current CT, Future FT). Refer to table 5.1 for population code key. Grey symbols represent above ground traits, whereas black symbols represent below ground traits. Symbols above the dashed 0:0 line indicate higher CVA under future conditions (FT).

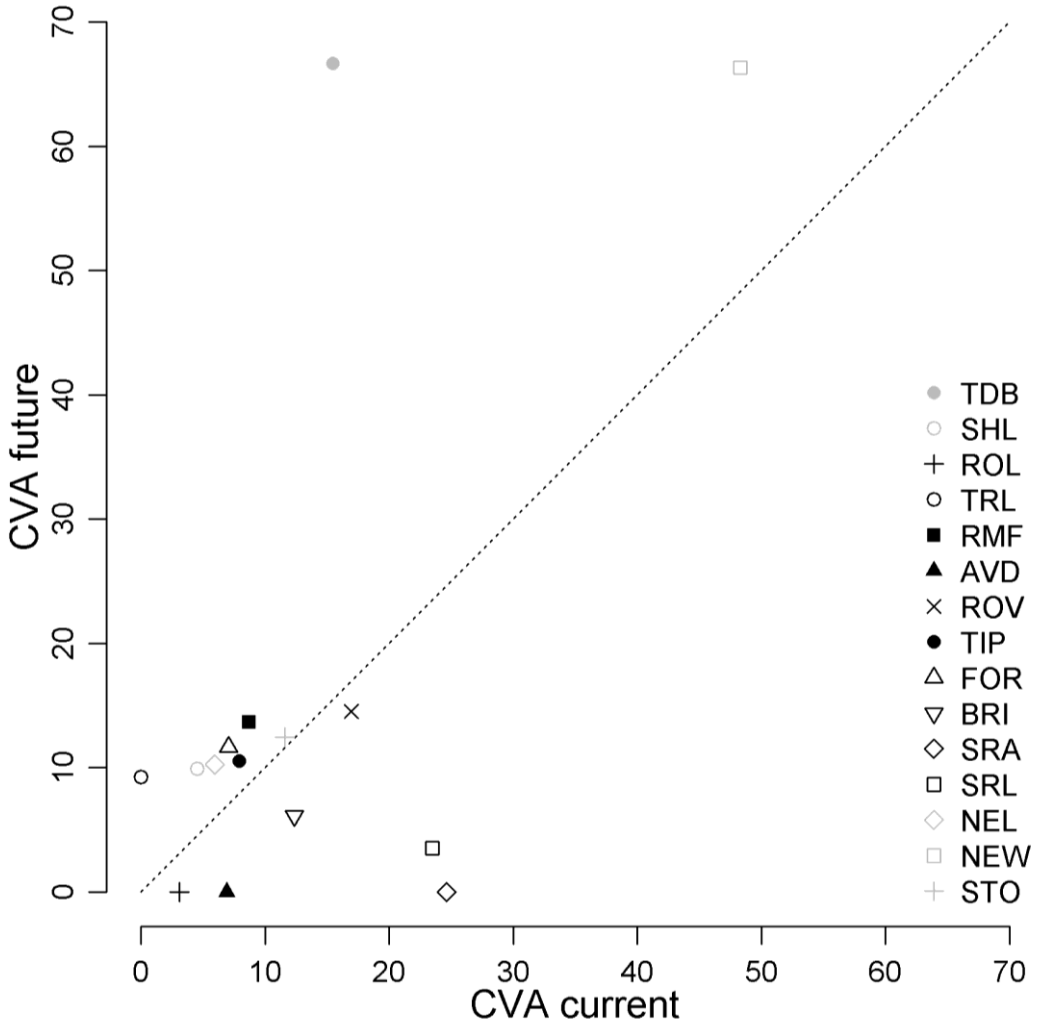


Table 5.5 Summary of early growth traits analyses by treatment and region. Model subsets were generated and compared by AICc to assess the importance of Treatment, Region, and their interaction as fixed effects. The best models were determined by AICc, and the presence (+) or absence (-) of fixed effects are indicated for each of the traits listed. ΔAICc values represent the difference in AICc between the null model and the best.

Trait	Mean CT	Mean FT	Treatment	Region	Treatment X Region	df	ΔAIC	AIC	V_{TREAT} (%)	V_{REG} (%)	$V_{TREAT\ X\ REG}$ (%)	V_{POP} (%)	V_{FAM} (%)	V_{BLOCK} (%)	V_{RES} (%)
Total Biomass (g)	0.05	0.38	+	-	-	6	36.47	1150.87	49.33	-	-	2.01	7.42	3.98	37.26
Shoot Length (mm)	17.16	64.15	+	-	-	6	33.00	1017.88	44.02	-	-	1.42	3.94	3.87	46.75
Root Length (mm)	300.26	435.57	+	-	-	6	28.70	343.71	37.45	-	-	2.83	4.22	1.86	68.04
Root Mass Fraction	0.42	0.43	-	+	-	6	2.24	-1141.74	-	1.78	-	0.78	0.00	3.67	93.81
Total Root Length (cm)	86.63	608.36	+	+	+	8	38.58	1089.16	22.74	0.096	27.83	2.29	6.14	3.69	37.21
Average Diameter (mm)	0.36	0.43	+	+	-	7	13.37	-1221.839	18.32	4.11	-	3.73	1.47	12.49	59.88
Root Volume (cm^3)	0.1	0.99	+	-	-	6	36.29	1255.13	49.63	-	-	2.40	5.23	3.94	38.79
Tips	267.24	1449.04	+	-	-	6	36.41	1054.13	48.27	-	-	1.43	6.71	3.76	39.83
Forks	370.54	2976.11	+	+	+	8	29.73	1147.13	21.35	0.15	26.81	2.15	6.19	8.68	34.67
Branching int. (cm^{-1})	3.04	2.63	+	+	-	7	23.61	829.05	9.97	1.93	-	0	0.84	1.01	86.24
Specific Root Area (m^2*g^{-1})	0.04	0.04	+	+	-	8	5.83	-2674	5.43	0.90	-	0.93	0.80	7.28	84.65
Specific Root Length ($m*g^{-1}$)	31.79	31.89	-	+	-	7	3.83	3264.37	-	4.73	-	1.40	2.67	6.85	84.35
Needle Length (mm)	17.9	33.37	+	+	-	7	46.64	979.25	45.12	-	-	1.84	7.46	1.81	43.76
Needle Width (mm)	0.81	1.001	+	-	-	8	29.18	-1084.23	19.12	1.41	-		7.92	2.45	69.10
Stomata Rows	3.99	4.73	+	+	-	7	26.64	-194.20	13.66	3.88	-	0.03	16.14	1.07	65.20

5.4.4 Relationship among traits

The number of stomatal rows was highly correlated with the total biomass (R^2=0.37, p<0.001) and the needle width (R^2=0.34, p<0.001), suggesting that the increase in stomata could be related to the increase in biomass in each of the plants. When we explored the relationship between the mean biomass of seeds and the mean biomass of seedlings at the end of the experiment (Fresh biomass) a small but positive correlation was detected (R^2=0.02, $d.f.$=4, p<0.01), but no differences among populations were found (all p-values >0.05).

5.5 Discussion

Despite the fact that our sampled populations were located in a relatively small geographic area at the north-western limit of the distribution of Scots pine, we were able to detect a substantial amount of intra-specific adaptive variation within and among populations and regions of this species, for which most traits were highly heritable. Although warmer conditions had strong effects on early growth traits by advancing germination and enhancing above and below-ground biomass by up to a factor of ten, there was a consistent response to warmer temperature in populations from both western and eastern Scotland, which was in contrast to our initial hypothesis. In addition, although cryptic genetic variation was present in the

majority of the traits under the novel warmer conditions, populations showed that the level of cryptic genetic variation did not vary among regions or populations.

5.5.1 Intra-specific adaptive variation

5.5.1.1 Seeds and emergence

Notable intra-specific variation among regions and populations was observed in seed viability and their emergence rates in our study populations. Although seed viability was high (between 61% and 70%) for all our Scots pine populations, it was lower for populations originating from the west compared to the east of Scotland, consistent with previous results (Mcvean, 1963). Seed viability, which is the primary factor determining the success of recruitment in forest tree populations, is believed to be controlled to a large degree by the temperature at the home environment (Reyes & Casal 2002), and the two are usually positively correlated (Kullman 2007). However, populations originating from western coastal areas of Scotland, which had lower values of viability, experience a significantly greater mean temperature in comparison with the eastern areas, which might be expected to favour seed viability, and the cause of this difference remains unclear.

Seed size is also believed to contribute substantially to seedling development, as it is generally accepted that seed size reflects the amount of nutrient reserves available to the embryo (Reich et al. 1994, Seiwa & Kikuzawa 1996). However, a contrast in the

contribution of seed size to development has been found in southern and northern populations of Scots pine. In Central and Southern European populations of Scots pine, a significant positive correlation was found between seed mass and seedling development (Reich et al. 1994), believed to be possibly only a temporary effect (Castro 1999; Righter, 1945), but the effect was not observed in northern populations (>55º in latitude; Reich et al. 1994). In our populations, however, we found a small but significant correlation among seed and seedling mass, suggesting that seed size might contribute to seedling development to some degree. Overall, Scottish seeds weighed less (5.63 ± 1.11mg) than those from the southern limit of the species distribution (9.21 ± 3.08 mg) (Castro 1999), which is assumed to be due to the shorter growing season and lower nutrient availability in northern populations (Reich et al. 1994). In addition, variation in the emergence ratio was observed among West and East populations, but variation within eastern populations (separated only by few kilometres) was greater.

5.5.1.2 Biomass and early growth traits

Overall, the proportion of total variation accounted for by within-population variation (variation among families) in biomass and above and below ground traits was greater than that attributable to among-region and among-population variation for most traits, but the latter were also significant. At the regional level, populations from the east showed a greater carbon allocation to roots (higher RMF) in

comparison to those from the west. Biomass allocation patterns are generally found to be influenced by local climate, particularly temperature and water availability. Thus, in natural conditions, cold and aridity have generally been found to promote greater biomass investment into roots (Luo et al. 2012), thereby enhancing the uptake of water and nutrients, which are more limited in such environments. Although a recent meta-analysis at a global scale attributed allocation shifts only to temperature (Reich et al. 2014), common garden trials indicate that plants that invest a greater proportion of biomass in their roots might have a selective advantage in arid conditions (Matías et al. 2014). Eastern Scottish populations experience both colder and drier conditions than those in the west. Therefore, our results suggest that individuals from colder conditions may achieve a selective advantage by being genetically predisposed to allocate more biomass to roots. Furthermore, under common garden conditions eastern populations not only invested more biomass in roots, they also developed a root system with a greater specific root length (higher SRL) based on thinner (lower average diameter) and more highly branched roots which would likely satisfy the need to explore deeper into the soil fraction. Similar results were reported by Comas & Eissenstat (2004) who found higher SRL, lower diameter and greater branching capacity in species needing stronger soil exploration and root defences, in this case in fast growing species. However, in our study, branching intensity of roots showed significant regional differences only during novel warming conditions (FT). Another very important selective force in Scotland is exposure to wind. In our trial, western populations had thicker roots than

populations from the east, a trait that has been shown to provide a better anchorage for the plant (Alvarez-Uria & Körner 2007). In the west, trees may be more exposed to strong west-south-westerly winds (Dore et al. 2006) coming directly from the Atlantic Ocean. Western populations could therefore be under selective pressure to produce individuals with a stronger anchorage system whilst the need to penetrate soil to find water is lower in populations originating from the west than the east. It is worth mentioning that we did not explore soil properties at the home sites, which may help to explain the results found. Other traits, such as SRA did not show significant variation between populations.

We also found consistent differences in some of the above-ground traits at the regional level. Therefore, populations from the west had greater numbers of stomatal rows than populations from the east. Similar results were found by Donnelly et al. (2016), who reported that stomatal density increased towards the West. The density of stomata on leaf surfaces *in situ* has been interpreted as a response to differences in moisture availability (Hogan et al. 1994, Brewer & Nuñez 2007) decreasing with reduced rainfall as an adaptation to conserve water in areas where there is less available. The density of stomata has also been suggested to decrease with increasing altitude (Hultine & Marshall 2000), presumably also assumed to be a water availability adaptation. Therefore, the differences observed in our common garden trial might indicate that the exposure of populations from the East to drier, higher altitude conditions may have driven adaptation in the form of

fewer stomatal rows. In addition, western populations showed also thicker needles in the CT, which showed a small but significant positive correlation with the number of stomatal rows.

5.5.1.3 Heritabilities and genetic components

All the differences that we observed in the phenotype during the experiment (described in the previous section) are due to the genotype, as we removed the environment by using common environmental conditions. The larger extent of within-population variation (family variation), which typically accounted between the 4 and 18% of the variance under current conditions (CT) with few exceptions (Table 5.4), was reflected in the substantial heritability values for most traits. Therefore, we observed moderate to high values of heritabilities ($0.18<h^2<0.49$ for CT) (but see SHL and RMR for CT), that were particularly large for total biomass or number of stomatal rows (0.77 ± 0.26 and 0.70 ± 0.26 respectively for CT), which indicate a significant variance in the phenotype is heritable, with the exception of RMF that showed no heritability under current conditions. Heritability estimates reflect the component of the phenotypic variation of the population that can be assigned to its genetic composition and is inherited from parental trees, and this can provide an indication about how much the trait can respond to selection. Furthermore, large estimates of heritability are usually associated with fluctuating natural selection (Bell, 2010; Donnelly et al. 2016). Therefore, temporal or spatial

variation (e.g. seasonal climatic variation or spatial heterogeneities) might have caused fluctuation in the selective forces acting on the studied Scottish populations, especially for stomatal rows and total biomass. We cannot disregard considerable standard errors - since high accuracy on those estimates can only be achieved with a very large sample size (Donnelly et al. 2016). Furthermore, some maternal effects might be present in our estimates (denoted by the small but positive correlation of seed mass vs. seedling mass), it seems that on balance the evidence indicates that growth traits are likely to retain substantial heritable adaptive capacity.

5.5.2 Response to temperature

As stated previously, temperature is an important factor influencing plant development (Hatfield & Prueger 2015) and is critical to all metabolic processes involved in uptake, release, and storage of carbon (McMahon et al., 2010). Therefore, earlier germination and increased growth under warming conditions is common for species inhabiting areas which are temperature-limited, as they are usually restricted by cold (Overdieck et al. 2007, Milbau et al. 2009, Pulkkinen et al. 2013). As expected, in our study we detected a strong role of temperature in substantially advancing seedling emergence by 10 days and dramatically enhancing biomass and above and below ground traits up to 10 fold, which was consistent in both west and east populations for most traits. The strong effect of the warmer temperature suggests temperature limitation for early growth in the study areas under current conditions.

Although the lowest temperature simulated in our experiment remained above the minimum temperature required for root growth (4-6ºC) (Alvarez-Uria & Körner 2007), it is likely that current temperatures (CT) are sufficiently low to slow the growth of both below and above ground traits. If this temperature limitation disappears as a consequence of predicted warming, it is likely to have consequences for the recruitment of the species in its northern distribution. Below, we discuss some of them.

5.5.2.1 Advance of germination

Emergence has been recognized to be a key stage that will set the context for subsequent development and natural selection (Verdú & Traveset 2005, Donohue et al. 2010). Therefore, earlier emergence in response to warmer future temperatures may result in a longer growing season providing seedlings with more time for development and establishment before the onset of adverse autumn conditions (Jones et al. 1997, Castro 2006, Richter et al. 2012a, Matías & Jump 2014). In addition, if warming is linked to adequate precipitation and does not cause resource limitation, it could increase tree metabolic processes in the subsequent seedling stages that, in turn, would lead to higher biomass accumulation (McMahon et al., 2010). Hence, our results, together with the predictions for Scotland, where increase of temperature is not expected to exceed the optimal and water availability is not predicted to decrease, suggest that warming and its consequent advancement of

emergence might have a consistent positive effect in early recruitment across the Scottish distribution. As a counterpoint, this positive effect does not remove the risk of late spring frost at higher latitudes, so seedlings emerging earlier in the growing season may be vulnerable to cold damage. The interaction between population and warming in emergence for BE(W), which germinated faster than the rest of the populations, could potentially be site-specific.

5.5.2.2 Biomass and above & below ground traits

The effect of warming was not only observed in emergence, but in most of the traits. However, warming did not cause shifts in carbon allocation biomass (RMF), except for the population BE(W), which shifted towards belowground biomass. Contrasting sensitivity of carbon allocation in response to temperature has been described previously, where greater biomass was allocated to shoots (Cerasoli et al. 2014), roots (Delucia et al. 1994, Domisch et al. 2001, Pumpanen et al. 2012, Matías et al. 2016) or with no change (Overdieck et al. 2007). Furthermore, for Scots pine, when the increase in temperature was combined with low water availability, a greater investment in roots was usually found (Richter et al. 2012b, Matías et al. 2016). Therefore, the lack of shift in biomass allocation for most populations in our study could be related to the absence of water limitation. However, it remains unclear why BE(W) was the only population that shifted towards belowground biomass,

although interestingly it was also the only population that showed an interaction for emergence under warming conditions.

Warmer temperatures had a strong effect on the below-ground system, with an increase in the length of roots, greater number of tips and forks as well as an increase in the root diameter. The growth of fine roots, which are primary organs for water and nutrient acquisition and are responsible for transferring resources between below-ground and above-ground parts, is usually positively correlated with temperature -below suboptimal limits- and also with nutrient uptake, root respiration and root mortality (Pregitzer et al. 2000). Hence, an increase in the root system under warmer conditions is not unusual. However, the amount of root length that was built per unit of root mass, as indicated by SRL did not change (except for BE(W)). This is in agreement with the results found by Alvarez-Uria & Körner (2007) who also reported a marked effect of temperature on root growth but lack of response of root quality, as reflected by SRL. This indicated that there was no short-term change in dry matter investment per unit of root length for soil exploration in cold vs. warm soil and is likely to be a reflection of the uniform nutrient supply provided to all plants. Interestingly, branching intensity, which governs exploration through the soil matrix and thus may also affect nutrient acquisition, was reduced under warmer conditions. Some authors have previously attributed the relation of higher root branching (Alvarez-Uria & Körner 2007) and higher root growth (Reich et al., 2014) with greater tolerance of cold environments.

Thus, the simulated warmer conditions (FT) might have favoured the reduction of root branching, which is in agreement with the adaptive response of investing less in roots and reducing branching in warmer western populations in comparison to eastern ones (significantly different at FT for BRI).

In terms of above-ground organs, an increase of stomata rows was observed under warmer conditions, and this was positively correlated with the increase in needle width. In contrast, some previous results have mentioned that warmer temperatures resulted in a reduction in stomatal numbers in recently collected individuals compared with those collected many years before and preserved as herbarium specimens (Beerling & Chaloner 1993) although this was also usually associated with an increase of CO_2 over recent decades. Levels of CO_2 were kept constant in our experiment. Number of stomata should be governed by a trade-off between obtaining sufficient CO_2 and water loss: higher CO_2 would imply less need for apertures so stomata density can be reduced. As CO_2 is stable in the control environmental facilities, warmer temperature but no changes in CO_2 might explain the increase in the number of stomatal rows. If the opportunity to reduce stomatal number is not provided, then there is still an impetus to increase numbers to obtain more CO_2 as metabolic potential (more tissue building possible) rises with temperature. Nevertheless, it seems plausible that confounding effects of temperature and CO_2 might be expected under more realistic warming conditions.

5.5.2.3 Impacts of genetic variation

Our results showed that the CVA estimates shifted under the warmer temperature treatment, however, traits did not respond homogenously to novel environments (i.e. FT in our experiment). In particular, CVA estimates increased in the majority of the traits under FT, but it declined for some root traits. Exposure to stress or novel conditions have the potential to release cryptic genetic variation (Hoffmann & Merilä 1999, Gibson & Dworkin 2004, Donnelly et al. 2016). The FT conditions might have therefore promoted the release of cryptic variation. However, the opposite response was found for some root traits and the reason for such trend remains unclear. We also found that western and eastern populations responded consistently to experimental warming (no interaction between temperature and region for most traits), which indicates that the level of cryptic genetic variation did not vary among regions or populations and similar capacities for phenotypic plasticity. Only RMF and FOR showed some interaction, but in the case of RMF it was the result of the shift of only one population BE(W). Similarly, the shift in heritabilities was not consistent, as it shifted either downward or upward for given traits under the warmer temperature treatment.

Overall, the results reported in this study suggest that a decline of the Scottish populations of Scots pine under a warmer climate due to poor seedling recruitment – emergence and early growth traits- seems unlikely. Although our results are

informative regarding the extent of phenotypic plasticity and adaptive potential in the populations, it is not possible to draw conclusions at the community level. Along with Scots pine, other species might benefit from warming, and the overall increase in success of Scots pine may not be realised if other species in the community also thrive. Furthermore, common garden trials and experimental set ups might prevent some natural disturbances, such us wind, spring forest, humidity diurnal variation or herbivore and pathogen attack, which might result in additional trade-offs not detected here (e.g. bigger early growth might be more vulnerable to wind and herbivores). Therefore, our results must be taken with caution, and more research is needed to better understand how adaptive responses in Scots pine might be modulated by interactions with other species and environmental factors not tested here.

5.5.3 Conclusions

In this study we investigated adaptive variation in early growth traits from western and eastern Scottish populations of Scots pine, and its response to projected climate conditions. We provide evidence to show that substantial levels of adaptive genetic variation are present in populations of Scots pine from both western and eastern Scotland. Adaptive genetic variation was usually larger within than among populations and regions, resulting in most traits being highly heritable, which might allow populations to genetically adapt to warmer conditions. We found some

evidence of cryptic genetic variation, especially in above ground traits, although all traits did not respond homogenously to novel environments. We found no evidence of differential response to warming among western and eastern populations - revealing that the level of cryptic genetic variation did not vary among regions or populations and a similar capacity for phenotypic plasticity under experimental warming. Therefore, if temperature predictions are met, it seems that early stages of Scottish populations of Scots pine are likely to benefit from warmer conditions. However, the added response of other species in the community and associated trade-offs, might determine the future of the adult populations of Scots within the community.

5.6 Acknowledgements

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5.8 Supplementary material

S5.1 Traits and indexes used in the analysis.

TRAIT/INDEX	**ABBREVIATION**	**DESCRIPTION**
Seed viability	SEV	Percentage of viable seeds (full seeds as a proxy of viable seeds) (%)
Seed weight	SEW	Mean weight of seeds (mg)
Emergence	EME	Day of emergence (day)
Survival	SUR	Percentage of survival (%)
Total Dry Biomass	TDB	Shoot Dry biomass + Root Dry biomass (g)
Shoot Height	SHL	Length of shoot (mm)
Root Depth	ROL	Depth of root (mm)
Root Mass Fraction	RMF	Root Dry biomass/Total Dry biomass
Total Root Length	TRL	Total root length (cm^{-1})
Average diameter	AVD	Average root diameter (mm)
Root Volume	ROV	Total Root Volume (cm^3)
Tips	TIP	Number of tips
Forks	FOR	Number of forks
Branching intensity	BRI	Number of tips/ Total root length (cm^{-1})
Specific Root Area	SRA	Surface area (winrhizo) / Root dry biomass ($m^{2}*g^{-1}$)
Specific Root Length	SRL	Total root length (winrhizo) / dry biomass root ($m*g^{-1}$)
Needle Length	NEL	Length of needle (mm)
Needle Width	NEW	Width of needle (mm)
Stomata rows	STO	Mean number of stomata rows in 5 needles

Chapter 6

General discussion

Photos in Chapter 6 main page taken from P. González-Díaz

6.1 Overview

The research presented in this thesis focuses on the ecologically and economically important tree species, Scots pine (*Pinus sylvestris L.*), the most widely distributed pine species in the world and a keystone species in areas of its distribution, such us in Britain. The aim of this research was to determine how different forces interact to shape and maintain within and among population genetic diversity of Scots pine from local to continental scales and how these might be impacted by future environmental changes. Two key approaches shaped the research undertaken. Firstly, we assessed neutral and adaptive genetic variation of Scots pine and the forces influencing such variation. In particular, we assessed historical and contemporary forces, such us climate and geographical variation together with human management that influence gene flow and selection and impact genetic diversity. Secondly, the study was undertaken at a range of spatial scales, investigating continental processes to regional and fine-scale within-population effects on the structure of Scots pine populations.

6.2 From legacies of the past to predictions for the future

The processes shaping historical migration and colonisation

Historical changes in climate influence the presence and absence of species over time (Davis 1986, Hewitt 2004) while recolonisation processes determine the amount and distribution of diversity in post-glacial populations (Hewitt 2003). The research in

chapter 2 has advanced our understanding of the dynamics of Scots pine historical migration and colonisation. In particular, chapter 2 used a novel approach combining genetic and palaeoenviromental data to cast light on the Holocene migrations and the forces shaping such migrations. The research presented in this chapter improves our understanding of both the spatial and temporal occurrence and associated genetic structure of Scots pine at the continental scale.

In **chapter 2** we observed how **historic climate changes** and **geographical barriers** have played an important role determining migration and colonisation routes and, as a consequence, in shaping the extent and spatial distribution of current genetic diversity of Scots pine at the continental scale. Our results provide greater resolution of the role of refuge areas in Holocene migrations of Scots pine and likely sources of colonisation. In particular, we agreed with most previous studies in the absence of contribution from populations originated in Italy and Asia Minor (Labra et al. 2006, Naydenov et al. 2007, Bilgen & Kaya 2007, Provan & Bennett 2008, Scalfi et al. 2009, Belletti et al. 2012, Dering et al. 2017) and confirm the contribution from Balkan populations to the central and northern European colonisation (Cheddadi et al. 2006, Prus-Glowacki et al. 2012). However, our results prompt a change in the view of the contribution of Iberian populations. We found some evidence to suggest a likely contribution of some Iberian populations to European colonisation, which in turn, might have contributed to the multiple origins of the most north-western populations, likely based on an admixture event between colonising fronts

originating from central-northern and south western European populations (i.e. northern Iberian or southern France) previous to the subsequent British migration.

The research in chapter 2 benefitted from a robust approach using multiple data sources in both species distribution modelling and phylogeographic analysis. Furthermore, the combination of both data analyses allowed us to dissentagle the effect of different climatic periods on spatial genetic structure, as the observed patterns may result from the interplay among processes acting at different temporal scales (Mayol et al. 2015). Specifically, each data analysis contributed in a different way, (i) palaeoenviromental data allow us to evaluate the effect of specific climatic conditions on neutral genetic diversity in a given period by testing each period separately, and (ii) phylogeographic analysis through a Bayesian framework allows us to estimate the time of the inferred demographic processes.

As species ranges have been and continue to be highly dynamic, the study of past forest change provides necessary historical context for evaluating the outcome of human-induced climate change and biological invasions (Petit et al. 2008). Therefore, the results obtained in chapter 2 have further developed our understanding about the historical forces shaping colonisation and its resultant genetic consequences. Those forces, such us geographic barriers and climate which determined the speed of migration, the existence of climate relicts, the likely existence of cryptic refugia, the presence of admixture events or successive bottlenecks, provide a better understanding to evaluate likely consequences of novel forthcoming conditions.

The role and processes shaping contemporary gene flow

Population structure isn´t static but dependent on changes in population size and connectivity over time and space (Hamrick et al. 1992). The research in **chapter 3** aimed to assess how **gene flow** and **genetic drift** interact to shape and maintain genetic diversity at a regional scale in the remaining natural fragments of Scots pine in the British Isles, which represent here the north-western limit of the vast Eurasian distribution.

Habitat fragmentation and destruction is recognized as one of the major threats to forest biodiversity (Lowe et al. 2005, Kramer et al. 2008). However, in chapter 3 we found that, despite historic reduction in abundance and the resulting geographical isolation of populations, high levels of neutral genetic diversity and low levels of population differentiation remain in the Scottish populations of Scots pine, with no evidence to suggest that any of the populations analysed here are genetically at risk. These findings suggest that effective population size, together with extensive gene flow and specifically wind-driven gene flow, has been high enough to limit the effects of range fragmentation and genetic drift. It has been previously argued that forest trees might show high genetic resilience in fragmented habitats (Hamrick 2004), and that despite potential barriers to gene flow such as population fragmentation (White et al. 2002, Petit & Hampe 2006, Wang et al. 2012, Davies et al. 2013) or phenological asynchrony, gene flow among populations can still be sufficient to counteract their genetic isolation. Our results confirmed such proposals.

In the context of recent findings of pollen asynchronies, we also detected that, gene flow patterns and geographic distribution of genetic variation were consistent with gene dispersal limitation due to prevailing wind patterns. This finding, together with the weak signal of isolation by distance among the Scottish populations, suggested that although some spatial limitation of gene dispersal occurs, gene flow is extensive and with a directional bias towards the East.

The role and processes shaping regeneration and establishment at the local scale

Anthropogenic impacts must be considered alongside geographical and climatic drivers in order to understand current population structures. Chapter 4 presents the first study in Scots pine addressing impacts of historic forest management practices on fine-scale spatial genetic structure. Our results provide evidence to show that although overall levels of genetic diversity in historically managed populations can be similar to unmanaged populations and as high as continental populations, spatial genetic structure can be considerably altered. Our research suggests that intense management practices that remove trees from the stand, such as felling, could alter fine-scale patterns of gene flow and increase genetic relatedness of individuals at fine scales with implications for inbreeding levels and, potentially, long-term adaptability. As a consequence, the extent of family clusters can be modified, as for instance in our study which increased up to 40 metres in managed sites compared

with almost no structure in unmanaged sites. Those results have implications for forest management strategies, such as seed collection strategies or spatial configuration of tree planting and selective felling.

Research in chapter 4 has developed our understanding of the early dynamics of Scots pine populations. The reduction of spatial genetic structure observed in juveniles following contemporary management to promote regeneration, indicates a high capacity of the species to recover after intense forest management. Those results highlight that ensuring large effective population sizes is key to successfully maintaining genetic diversity in Scots pine.

The use of a medium-high number of individuals within populations and cohorts (N=57-181 in our study) provided power in our research to detect fine-scale spatial genetic structure, which has been previously acknowledge as a prerequisite to obtain reliable inferences in these spatial analysis (Cavers et al. 2005, Jump & Peñuelas 2007). However, an in-depth representation within stand and cohort was a trade off against the capability to increase site replication. Although we obtained a consistent pattern in managed stands, and a pattern in unmanaged stands comparable with natural stands in other studies, ideally a greater representation of sites would be assess in the future to generalize the genetic spatial trend of Scots pine in a wider context.

The role and processes shaping natural selection

Implicit in the idea of changes on distribution over time is that, individuals are subject to different conditions over space. Given high diversity, selection is also a fundamentally important component of contemporary population structure. The research in chapter 5 aimed to characterize the **intraspecific adaptive variation** in Scots pine early growth traits at a regional scale, from populations originating from contrasting environmental conditions within Scotland and to estimate the likely response of those populations to projected **climate change.**

In **chapter 5** we found that substantial levels of adaptive genetic variation are present in the Scottish populations of Scots pine within and among populations and regions. This finding is likely a result of **selective processes** resulting from the different **environments** they live in, in combination with high trait heritability. Previous studies have shown that a very large proportion of variability lies within populations, both at neutral (Forrest 1980, Kinloch et al. 1986, Provan et al. 1998, Wachowiak et al. 2011) and at the adaptive variation levels (Perks & Mckay 1997, Salmela et al. 2013, Donnelly et al. 2016) and our findings confirmed this pattern.

Chapter 5 presents the most comprehensive research of Scots pine adaptive diversity response to predicted temperature conditions in Britain. We observed that warmer conditions had strong effects on early growth traits by advancing germination and enhancing above and below-ground biomass by up to a factor of ten. However, we

observed that there was a consistent response to warmer temperature in populations from both western and eastern Scotland. Therefore, our results did not support differential levels of cryptic genetic variation among western and eastern Scots pine populations. Consequently, if temperature predictions are met, it seems that early stages of Scottish populations of Scots pine are all likely to benefit from warmer conditions.

Using controlled environment experiments enabled us to exclude environmental variation, and determine how much variation is genetically determined. However, controlled environment studies also exclude other factors, such us the added response of other species in the community and associated trade-offs which, if included, might determine the future of the adult populations of Scots within the community. In the future, it would be desirable to explore other factors influencing the response of early traits under forthcoming environmental conditions.

The large within-population representation was again a trade off against the capability to increase site number. This compromise was determined to represent an in-depth sampling scheme within populations, to have a greater resolution to explore processes acting at local or regional scales (Donnelly et al. 2016).

6.3 General synthesis

In this thesis a combination of observational and experimental approaches has shown that within population diversity (both neutral and adaptive) is high in the studied Scots pine populations, and higher than levels of among population variation, which is usual in trees (Forrest 1980, Kinloch et al. 1986, Provan et al. 1998, Wachowiak et al. 2011, Perks & Mckay 1997, Salmela et al. 2013, Donnelly et al. 2016). Taken together these findings from adaptive variation (Chapter 5) and neutral genetic variation (Chapters 2, 3 and 4), show that although gene flow may counteract genetic drift and restrict local adaptation, adaptive divergence may still take place under strong selective pressure. This assumption agrees with previous assumptions (Savolainen et al. 2007) and supports previous studies that suggest that adaptive divergence might have occurred in the Scottish populations of Scots pine (Salmela et al. 2013, Donnelly et al. 2016).

The research presented in this thesis has shown that the movement of Scots pine through the continent and at a regional scale has been largely driven by natural processes (Chapter 2, 3 and 5). However, long-standing human impacts have modified the spatial configuration of populations with no changes in overall levels of genetic variation (Chapter 3 and 4), but modification of local patterns of genetic variation (Chapter 4).

By using data from different spatial scales, we were able to observe patterns

resulting from the interplay among processes acting at different temporal scales. Therefore, in chapter 2, continental data and the combined used of palaenvironmental and phylogeographic analysis allowed us to detect historical forces acting during the last period of major climate change. Complementary to this work, regional genetic data in Chapter 3 allowed us to detect contemporary processes influencing neutral genetic diversity. Furthermore, the use of different cohorts in Chapter 4 also allowed us to disentangle processes acting at different temporal scales. Adults showed processes acting in the recent past (i.e. when they first established, approximately 200 years ago), whereas juveniles served as interpreters of the present. As a consequence, adults identified that historical forest management practices have influenced fine-scale spatial genetic structure of Scots pine stands, whereas juveniles showed a high capacity to recover after disturbance. Finally, by using experimental conditions, we were able to project future conditions, and to evaluate the effect of current and likely predicted climate on early growth at regional scales.

Contemporary local dynamics shaped the genetic layout of regeneration in managed stands in chapter 4, suggesting a high capacity of Scots pine to recover after disturbances. This finding, together with the strong effects of predicted temperature on early growth shown in chapter 5, might have implications for forest resilience and establishment such as the creation of abundant and diverse recruitment banks which might maximize their potential to adapt to a changed environment. This

potential will be particularly important in the context of climate change (Millar et al. 2007, Hoffmann & Sgrò 2011, Alfaro et al. 2014, Fady et al. 2016) under which selection pressures are expected to change and highlights the importance of the conservation of genetic sources.

6.4 Future research and conclusions

By exploring interactions between climate, geographical variation and human activity, our results help to further disentangle the forces maintaining genetic diversity in one of the most widespread conifers in the world. The research in this thesis improves our understanding of impacts of migration and selection on Scots pine and will allow us to refine predictions of the impact of future range shifts in response to contemporary climate changes. Our findings have practical application for forest management and implications for future research, as outlined below.

(1) This research highlights the importance of maintaining large effective population sizes (Chapter 3 & 4), especially in geographically marginal populations, to increase the probability of forest persistence.

(2) Bias in gene dispersal due to prevailing wind patterns and the likely landscape impacts on genetic diversity in the Scottish populations should be taken into account when designing afforestation strategies or determining priorities in conservation and management plans.

(3) Although there was no evidence to suggest that any of the populations analysed here are genetically at risk, the extensive establishment of Scots pine plantations, at least in Scottish lands, based upon a system of clear felling and artificial regeneration (Mason 2000) might alter local patterns of gene flow leading to impacts in the diversity and structure of subsequent generations of native Scots pine. Consequently, future monitoring of both neutral and adaptive genetic diversity should focus on addressing levels and structure of genetic variation in Scots pine plantations and surroundings. The monitoring of genetic diversity could follow the schemes mentioned in chapter 1 (Graudal et al. 2014, Aravanopoulos et al. 2015).

(4) Guidelines for Scots pine seed collection should aim for minimum sampling distances between mother trees of at least 40 metres, in order to ensure a broad sample of genetic variability.

(5) The study of the spatial component of genetic diversity alongside tree demographic structure can help to detect both historical and contemporary effects of disturbances in tree populations, which is highly recommended when evaluating the effects of forest management practices. The evaluation of successional change is also essential to properly assess genetic dynamics within populations and to adequately detect early responses to forest management practices.

(6) Although chapter 5 suggested that, if temperature predictions are met, early stages of Scottish populations of Scots pine are likely to benefit from warmer conditions, the added response of other species in the community and associated

trade-offs, might determine the future of the adult populations of Scots within the community. Therefore, we recommend exploration of other forces and processes that might influence early growth.

(7) In chapter 5 we evaluated early growth traits during the first growing season, however, long-term experiments among native Scots pine forest from Scotland to explore the likely consequences of climate change and the evidence and extent of adaptive divergence would be desirable.

In summary, although a considerable amount of prior research has focussed on phylogeography and descriptive analyses of the levels and structure of neutral genetic variation, together with provenance trials, the work presented here makes a specific advance in the following areas: (i) continental processes during Holocene migrations (ii) contemporary fine-scale processes, (iii) historical and recent management practices influence, (iv) processes acting within and among populations and (iv) potential responses to forthcoming environmental conditions. These findings leave us with a better understanding not only into the evolutionary aspects of Scots pine but the potential consequences of the combination of contemporary human and environmental factors. The findings of this thesis can help to make better management decisions in the future of Scots pine, with the aim to preserve both neutral and adaptive variation at global and local scales and safeguard Scots pine forest diversity for future generations.

6.5 References

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