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A population genetic study of Pasqueflower

In situ and Ex situ Conservation Genetics of a Vulnerable UK Plant Species

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Abstract

The population genetic structure of the vulnerable UK plant species *Anemone pulsatilla* L. reflects geographic patterns of historical range fragmentation and the influence of population decline and restoration intervention. Positive spatial auto-correlation of natural in situ populations of *A. pulsatilla* lends support to a scenario for genetic drift (i.e. random drift of allelic frequencies) driving the emergence of population genetic structure as a consequence of fragmentation. Multivariate and STRUCTURE analysis estimates the partitioning of genetic variation among four natural population genetic clusters (broadly defined by geographical regions of the species' range) and a fifth, highly differentiated, genetic cluster defined by introduced genotypes of unverifiable genetic origin to the casually augmented AN population. It is recommended that restoration intervention (i.e. to augment declining populations or introduce populations to enhance gene flow) source propagules for introduction from within the local population genetic cluster in order to maximise the potential for introduction/exchange of locally adaptive genetic variation.

The existing ex situ gene conservation strategy for *A. pulsatilla* can be predicted to under-represent the species' natural genetic variability due to limited sampling effort. At a minimum, a representative ex situ gene conservation strategy for the safeguard of *A. pulsatilla* UK variability should aim to capture representative accessions from the most diverse population/s of each of the four natural population genetic clusters. It is also recommended that the six native AN genotypes are sampled for ex situ conservation due to a disproportionately high level of unique genetic variation. A pilot study of regenerated ex situ accessions supports a prediction that the following factors act on genetic diversity: (a) survivorship; (b) number of generation removed from the wild; (c) effective population size.

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1 Introduction

1.1 The Value and Vulnerability of Global Plant Diversity

1.1.1 A Contemporary Biodiversity Crisis

It is widely acknowledged by the international scientific community that the world is approaching a global biodiversity extinction crisis (Thomas *et al.* 2004; Chivian & Bernstein 2008; IUCN 2009; SCBD 2014). Whilst the balance between the rate of extinction and evolution of species has always existed in flux, the current rate of species loss is estimated to be greater than the natural background rate with the potential to approach a mass extinction event (Ricketts *et al*. 2005; Thuiller 2007; IUCN 2013). The magnitude and impact of a contemporary biodiversity crisis is likely to vary among geographical regions, habitats and human communities (Hawkins *et al.* 2008). However, the widespread ecological consequences of such as extinction crisis are potentially catastrophic, the economic consequences incalculable, the cost for human culture, enterprise, life and well-being, irreversible (SCBD 2014).

1.1.2 Ecological and Economic Contribution of Plant Diversity

As the fundamental basis for the world's terrestrial ecosystems the current and projected future decline in vascular plant diversity represents perhaps the most urgent point of conservation action to emerge from the contemporary biodiversity extinction crisis (Hawkins *et al.* 2008; Ellstrand & Elam 2011; Sharrock & Wilson 2014). As the physical and trophic scaffolding of natural, semi-natural and farmed environments vascular plant communities provide vital ecological services for humanity (Hawkins *et al.* 2008; Sharrock &

Wilson 2014). Both directly, through the exploitation of raw plant materials, and, indirectly, by supporting vital environmental and ecological processes (Hawkins *et al.* 2008).

Natural plant products provide nutrition, medicine, clothing, fuel, and timber products for even the most advanced human communities (Hawkins *et al.* 2008). Some 80% of the world's population depend on local medicinal plants for their primary health care whilst a significant proportion of the dietary micro-nutrients consumed globally, essential to maintaining health and well-being, are sourced from wild plant species (Schuster 2001; Hawkins *et al.* 2008; Sharrock & Wilson 2014). The survival of much of the world's rural community is intimately linked to the local availability of wild plants, to meet basic living requirements (for food, shelter, cooking and heating fuel etc.) and to provide sustainable livelihoods. For example, over 60 million hectares of land is currently registered for wild plant collection (the large majority based in developing economies) in support of the production of a variety of organic products for local and global distribution (SCBD 2014b; Sharrock & Wilson 2014).

Plant diversity serves a unique role in supporting productivity of natural and managed ecological communities, the sequestration of atmospheric carbon and cycling of soil nutrients support environmental stability whilst complex species interactions maintain vital ecosystem services such as pollination and pest control (Ellstrand & Elam 2011; Hawkins *et al.* 2008). Natural plant diversity also provides an important resource for future agricultural crop development as a genetic basis for disease resistance and extreme environmental tolerances are likely to become increasingly important traits for agricultural species (GCDT 2013; Sharrock & Wilson 2014). Forest and grassland ecosystems are increasingly recognised to be of particular significance in the provision of ecosystem

services, and a genetic resource, vital to the sustainability of human communities across the globe (White *et al*. 2000).

1.1.3 Global Status of Plant Diversity

Natural plant communities, and the ecosystems they support, are fundamental to the development and maintenance of human society and culture. It is of concern, therefore, that the political momentum, and conservation strategy, required to halt the continued and accelerating decline in global plant diversity has long been under-represented within the international biodiversity agenda (Ellstrand & Elam 2011; Sharrock & Wilson 2014). The inadequate representation of conservation strategy for global plant diversity is due, in part, to the difficulty of gaining a comprehensive account of the world's floristic diversity (Hawkins *et al.* 2008; Sharrock & Wilson 2014).

At the latest estimate, known global plant diversity stands at approximately 350, 000 species with 10% to 20% of extant plant species thought yet to be discovered (Paton *et al.* 2008; Joppa *et al.* 2011; Scheffers *et al.* 2012). Published in 2010 the 'Plant List', produced collaboratively by RBG Kew and the Missouri Botanical Gardens, represents the first internationally endorsed collation of accepted plant names for the c., 350,000 known plant species (The Plant List 2013). Whilst this inventory of known plant species diversity represents an important first step in assessing the extent, distribution, and status of global plant diversity it is by no means a comprehensive conservation tool. Further effort is required to update the Plant List as new species are discovered and named (at a current rate of approximately 2000 species per year) and, critically, to produce by 2020 a World Flora Online (WFO) (BGCI, GSPC & SCBD 2014; IPNI 2014). When complete the WFO will provide botanical descriptions, distributions, and up to date threat assessments for all known plant

species and will act as an invaluable information resource for the development of effective national and international plant diversity conservation strategy (BGCI, GSPC & SCBD 2014).

Assessment of the conservation status of the world's known plant species is incomplete and progress lags behind that made for other taxonomic groups, just 6% of plant species have been assessed under internationally accepted criteria of the IUCN Red List (Sharrock & Wilson 2014). The updated IUCN red list of threatened species, published in 2013, identifies 10, 065 threatened plant species for which the conservation status has been assessed under internationally accepted guidelines (IUCN 2014). A broad assessment of the conservation status of the world's flora 'A Sampled Red List Index for Plants', conducted by RBG Kew and the Natural History Museum (London), indicate that 20% of all extant plant species are currently threatened with extinction (RBG Kew 2010).

Whilst efforts to secure a comprehensive understanding of global plant diversity and conservation status are ongoing a clear picture has emerged of the great disparity in distribution of plant species richness. Occupying just 2.3% of the earth's terrestrial surface global 'biodiversity hotspots', defined by high levels of species richness, are thought to account for half of all endemic plant species many of which are yet to be discovered (Joppa *et al.* 2011). Islands account for almost half of the world's 'biodiversity hotspots' while tropical forests are the predominate habitat (Branwell 2007; Novotny *et al.* 2007; Vié *et al.* 2009). Tropical forest habitats are subject to high levels of exploitation of natural plant resources whilst the ecosystems supported by island habitats are sensitive to environmental change and/or habitat disturbance (Laurance *et al.* 2001; Hawkins *et al.* 2008). The most significant resources for natural plant diversity globally are therefore placed at high risk of plant species extinction as a consequence of human driven pressures on the natural

environment. Mega diverse countries that host a large proportion of these at risk 'biodiversity hotspots' have therefore become a focus for the international plant conservation community (Sharrock & Wilson 2014).

1.1.4 Current and Future Threats to Plant Diversity

Human driven habitat loss and degradation is widely acknowledged to be the primary deterministic factor underlying the contemporary decline in global plant diversity with the result that 4 out of every 5 endangered plant species is directly affected by habitat loss (Young & Clarke 2000; Vitt & Havens 2004; IUCN 2009; BGCI 2014). It is estimated that by 2032 more than 70% of the world's terrestrial habitats will be disturbed or destroyed; 40% of the world's forest, a significant habitat for global plant diversity, has already been lost to timber exploitation or clearance for agriculture and other development (BGCI 2014). Whilst 10% of the world's surface has been awarded a protected status the value of these areas for the conservation of plant diversity is difficult to assess due to a general lack of information regarding the management of these areas, the representation of different habitat types, and the representation of endemic plant species (Hawkins *et al.* 2008). Additional human mediated factors that directly and significantly impact on plant diversity include the introduction of competitive non-native plant species and disease to natural ecosystems, pollution, and the commercially driven over exploitation of plant resources (BGCI 2014; Sharrock & Wilson 2014).

Climate change and associated, potentially devastating, habitat and ecosystem disruptions represent perhaps the greatest challenge to the future conservation of global plant diversity (Thomas *et al.* 2004; Hawkins *et al.* 2008; Corlett & Westcott 2013). It has, in recent years, become accepted by the wider scientific community as irrefutable that global

temperature is rising at an unprecedented rate, as a direct result of human activity (Hawkins *et al*. 2008). A range of climate change scenarios predicting global temperature increases between 1.8 ^{o}C and 6 ^{o}C by the end of the current century and are considered valid by the International Panel for Climate Change (IPCC) (Hawkins *et al*. 2008; IPCC 2014).

Plant species particularly vulnerable to the environmental impact of climate change include those that are already experiencing population decline and range contraction as a result of habitat loss or disruption. Endemic and restricted range species (such as island species) and those species with low dispersal ability and/or complex ecosystem associations are predicted to be highly sensitive to the environmental shifts associated with climate change (Hawkins *et al.* 2008). Such species are limited in their ability to adapt to environmental or ecosystem change or to track the fluctuating distributions of suitable habitat within shifting climate envelopes (Thomas *et al.* 2004; Hawkins *et al.* 2008; Corlett & Westcott 2013).

A conservative estimate of a 2 ^{o}C rise in global temperature over the next 100 years is expected to increase the proportion of threatened plant species to 50% global plant diversity by the end of the century (Bramwell 2007). Should global temperatures rise by 4 ^{o}C (the mid-range of reasonable projections) the impact on plant diversity, and accompanying economic and humanitarian costs, become incalculable as viable conservation management options are limited by the extremity of environmental change (Hawkins *et al.* 2008).

1.2 Population Genetics of Threatened Plant Species

1.2.1 Fragmentation

Threatened plant species often have fragmented distributions that are a remnant of a former more continuous and extensive range, extant populations are typically small and highly dispersed occupying a reduced range extent (Loveless & Hamrick 1984; Karron 1987; Gibbs 2001; Frankham 2003; Rosetto 2006). Stochastic (random or chance) selection factors are predicted to exert a disproportionate influence on the genetic diversity of fragmented populations (as opposed to directional natural selection factors) with the consequence that adaptive genotypes may be lost from the population by chance (Luikart *et al.* 2003). Gene flow among fragmented populations is typically low with the result that there is little opportunity for re-introduction of lost adaptive genetic diversity (Rosetto 2006). Such detrimental population genetic processes, characteristic of fragmented populations, are predicted to accelerate a threatened species decline towards extinction, even in the absence of further habitat loss or in the case of habitat restoration occurring too late for a species recovery (Ouberg *et al*. 2010; Sherwin & Moritz 2000; Frankham *et al.* 2002).

1.2.2 Genetic Drift

Within Population Genetic Diversity

The process by which stochastic selection factors influence the genetic diversity (allelic frequencies) maintained within populations is termed genetic drift (Sherwin & Moritz 2000; Rosetto 2006; Frankham 2010). Whilst every natural (finite) population experiences genetic drift the effects on within population genetic diversity become more pronounced as population size decreases and isolation increases (Luikart *et al.* 2003; Ellstand & Elam 2011).

Rare and less frequent alleles (gene copies) are vulnerable to loss from small populations through the random sampling processes of genetic drift (Rosetto 2006). In the absence of gene flow to reintroduce rare alleles, genetic drift is predicted to lead to a continual decline (erosion) of adaptive genetic diversity within fragmented populations. Declining within population genetic diversity has frequently been observed within fragmented populations of threatened plant species and there exists strong empirical and theoretical support for the role of genetic drift as a key driver of this decline (Prober & Brown 1994; Young *et al.* 1996; Sherwin & Moritz 2000; Rosetto 2006; Frankham *et al.* 2002).

Among Population Genetic Variation

As a stochastic process, genetic drift is predicted to affect the genetic diversity (allelic frequencies) of each population of a fragmented species differently. Genetic drift is therefore predicted to drive among population genetic differentiation, i.e. the partitioning of genetic variation among fragmented populations (Young *et al.* 1996; Sherwin & Moritz 2000; Frankham *et al.* 2002; Allendorf *et al.* 2013). Habitat and species' range fragmentation is typically a gradual, incremental, process and therefore genetic distance (variation of allelic frequencies) among populations is expected to correlate with geographic distance (as a proxy measure of time since fragmentation) (Sherwin & Moritz 2000; Frankham *et al.* 2002; Allendorf *et al.* 2013).

1.2.3 Founder Effect

The founder effect refers to the random sampling of genomes from a wider population during a dispersal or fragmentation event. The founder effect is understood to act as a stochastic selection factor influencing the genetic diversity (allelic frequencies)

maintained within fragmented populations and directing the partitioning of genetic variation among populations, with the effect intensifying for small and/or edge of range populations (Frankham *et al.* 2002; Ellstrand & Elam 2011).

1.2.4 Inbreeding

Inbreeding, the breeding of close relatives, is an inevitable consequence of species' range fragmentation as population size and connectivity declines over time. Inbreeding acts to increase the probability that an individual will be homozygous (the inheritance of alleles identical by descent from both parents) for any given gene loci (Sherwin & Moritz 2000; Frankham 2003; Rosetto 2006). The consequence of inbreeding is therefore a decline in the allelic diversity of individual genomes, the genetic diversity maintained within a population may however be unaffected (Sherwin & Moritz 2000; Frankham 2003).

1.2.5 Genetic Diversity and Extinction Risk

'As the raw material of natural selection, the conservation of genetic diversity is understood to be integral to maintaining evolutionary potential, and therefore a species long-term viability (Frankham *et al.* 2002; Allendorf *et al.* 2013). As discussed, fragmented populations are predicted to experience declining within population adaptive genetic diversity, as a consequence of the random genetic sampling processes of genetic drift and the founder effect (Sherwin & Moritz 2000; Rosetto 2006). The stochastic genetic sampling effects inherent to species' range fragmentation are therefore predicted to lead to a decline in potential for remnant extant populations to respond adaptively to local environmental pressures.

An association between small population size and declining genetic diversity is well supported both empirically and theoretically although the relationship between genetic diversity, evolutionary potential, and extinction risk has proven more problematic to elucidate (Frankham 2003. Ouberg *et al* 2010). Circumstantially, the link between reduced genetic diversity and heightened extinction risk is demonstrated by the observation than endangered species in general have lower genetic diversity than non-endangered species (Frankham 1995). Experimentally, plant populations with lower genetic diversity display a greater extinction rate than those with higher genetic diversity measures (Newman and Pilson 1997). In natural plant populations the most unequivocal evidence for the relationship between loss of genetic diversity and heightened population extinction risk arises from studies of the self- incompatibility locus (Les *et al.* 1991; Demauro 1993; Young *et al.* 2000: Frankham 2003).

1.2.6 Inbreeding Depression and Extinction Risk

High levels of inbreeding can be expected to lead to declining population fitness, i.e. inbreeding depression, as a result of the accumulation of individuals homozygous for recessive, deleterious, alleles (Frankham 2003). Potentially therefore, increased levels of inbreeding could be predicted to result in a heightened population extinction risk through loss of individual survival and reproductive potential. There is clear evidence, from both experimental and wild systems, that inbreeding adversely impacts on the fitness and viability of natural populations (Oubourg *et al.* 2010; Frankham 2003).

1.2.7 Conservation Genetic Management of Threatened Species

'Adequate genetic management of fragmented populations is rare, and one of the greatest unaddressed issues in conservation biology' (Frankham 2003)

Essentially an applied science, conservation genetics aims to advance understanding of genetic issues in biodiversity conservation and translate these concerns into practical approaches for the conservation of threatened species (Frankham 2010). Key population genetic concerns for conservation managers include: (a) how to define management units within species based on the distribution of genetic variability among populations; (b) the population size and genetic diversity required to maintain long-term population viability; (c) when to consider population augmentation or reintroduction as a beneficial conservation strategy to restore adequate levels of genetic diversity; (d) how to source and introduce potential colonists (Latta *et al.* 2008); (e) and, how to assess and minimise the risk of outbreeding depression in the course of restoration intervention – i.e. the swamping of locally adapted genotypes by introduced genetic material potentially resulting in a decline in fitness of the second generation (Frankham 2010).

1.2.8 Molecular Genetic Tools for the Study of Conservation Genetics

Neutral Genetic Marker Studies

The emergence of conservation genetics as an empirical science has been reliant on advances in evolutionary and molecular genetic techniques. Quantifying the genetic and associated fitness consequences of species fragmentation provides a fertile field for scientific research. Whilst variation in Quantitative Trait Loci (QTL's), genetic markers that relate directly to fitness characters (such as survival and fertility), are of the most

significance to conservation practitioners these regions of the genome are the most complex to identify and analyse (Frankham *et al.* 2002). Conservation genetics is primarily concerned with understanding the impact of fragmentation on species population genetic structure, i.e. the distribution of genetic variation among populations (Frankham *et al.* 2002; Rosetto 2006). Genetic markers derived from neutral areas of the genome are freed from the filtering effects of natural selection. Therefore, variation of neutral marker allelic frequencies among fragmented populations will reflect the action of stochastic genetic sampling factors, such as genetic drift and founder effect, on population genetic structure (Sherwin & Moritz 2000; Vilas *et al.* 2005; Frankham 2010; Ouberg *et al.* 2010).

Microsatellites: Highly Polymorphic Neutral Genetic Markers

The most informative neutral genetic markers are those that show high levels of polymorphism (allelic diversity) within populations and are therefore able to record fine scale variation in allelic frequencies between individuals and among fragmented populations (Frankham *et al.* 2009). Microsatellite markers, areas of the genome composed of tandem DNA motifs (repeat units typically 1-5 base pairs in length), are generally highly polymorphic within populations (Hoglund 2009). Frequent errors in the DNA replication phase result in the insertion or deletion of a repeat unit within the microsatellite region and this leads to the creation of a new (selectively neutral) mutation to the population. Microsatellite motifs occur in neutral regions of a plants nuclear genome (bi-parental inheritance) and the plastid genome (maternal inheritance). The plastid genome is more highly conserved, i.e. displays less variation, than the nuclear genome among generations. Therefore, plastid genome microsatellite markers are informative on the ancestral relationships of populations and can be applied to trace historical range dynamics (Hedrick 2005). Nuclear genome microsatellite

marker studies are most appropriately applied to explore the population genetic processes associated with recent fragmentation events (Frankham *et al.* 2002; Rosetto, 2006; Sherwin & Moritz 2009).

Limitations of Neutral Genetic Marker Studies

A key application of conservation genetic research is to translate neutral genetic marker studies of population genetic structure (distribution of genetic variation among fragmented populations) into effective conservation strategy (Henry 2006; Sherwin & Moritz 2000; Frankham 2010). For example, the identification of genetically distinct population clusters (differentiated allelic frequencies) that can define a discrete management unit, and the identification of populations perceived to be at genetic risk and requiring intervention to introduce viable levels of genetic diversity (Frankham 2010).However, there are, as yet unresolved, questions as to the appropriateness of applying studies of neutral genetic diversity to inform management decisions for the maintenance and restoration of species adaptive genetic diversity and evolutionary potential (Frankham 2003; Oubourg *et al.* 2010).

Whilst there are practical difficulties in identifying and developing genetic markers for adaptive trait loci (QTL's), studies that have explored the validity of using F_{ST} (a measure of the partitioning of neutral genetic variation among populations) as a proxy measure for Q_{ST} (a measure of the partitioning of adaptive genetic variation among populations) have demonstrated no consistent, predictable, relationship between these two measures of population genetic differentiation (Reed & Frankham 2003). Caution should be applied therefore in inferring fitness costs, and increased extinction risk, from observations of

neutral genetic marker studies which record declining genetic diversity, and increased among population variation, without identifying co-varying phenotypic traits.

1.3 A Global Strategy for Plant Conservation (GSPC)

1.3.1 Global Strategy for Plant Conservation: Vision

The urgent need for a coordinated international response to the current unprecedented rate of decline in global plant diversity was acknowledged by the United Nations Convention on Biological Diversity (CBD) in 2002 with the establishment of the Global Strategy for Plant Conservation, GSPC (Jackson & Kennedy 2009; Li & Pritchard 2009). The broad vision of the GSPC is to '*halt the continuing loss of plant diversity*' in recognition of the unique and integral ecological role played by natural plant diversity in supporting biodiverse habitats and sustainable human communities (SCBD 2011, Sharrock & Wilson 2014). The significance of the GSPC is that for the first time a series of explicit, outcome orientated, international targets (16 in all) establish a clear pathway towards facilitating an understanding, conservation, and sustainable use of the world's plant diversity (Jackson & Kennedy 2002; BGCI 2014; Sharrock & Wilson 2014). The official UK response to the GSPC is the 'Plant Diversity Challenge', launched in 2004, representing a partnership between Plantlife International, The Joint Nature Conservation Committee and Royal Botanical Gardens (RBG) Kew.

1.3.2 Global Strategy for Plant Conservation: Progress

The GSPC has been hailed as one of the most successful outcomes of the Convention on Biological Diversity (BGCI 2010). Achievements in progressing understanding of global plant diversity include internationally collaborative projects such as the Plant List and World

Flora Online. Achievements in progressing conservation of global plant diversity include a significant expansion of global capacity for ex situ conservation (i.e. maintenance of species outside of the natural environment) as a safeguard for crop and wild plant genetic resources (BGCI, GSPC & SCBD 2014; Sharrock & Wilson 2014). Progress has been slow however towards achieving international targets for the sustainable use of natural plant resources and for the in situ conservation (maintenance of species within the natural environment) of global plant diversity (BGCI, GSPC & SCBD 2014).

1.4 Global Targets for the Ex situ Conservation of Plant Diversity

1.4.1 Facilities for the Ex situ Conservation of Plant Diversity

Ex situ conservation is defined in Article 2 of the Convention on Biological Diversity, 1992 as *'the conservation of components of biological diversity outside of natural habitats'* (Thorman *et al.* 2006). Whilst it is widely acknowledged that the highest priority is to conserve populations of threatened plant species in situ, where they support ecosystem functioning and contribute to biological diversity, it is increasingly recognised that ex situ collections potentially have an important, complimentary, role to play in the conservation of global plant diversity (Falk & Holsinger 1991; Guerrant *et al.* 2004; Li & Pritchard 2009; Ellstrand & Elam 2011; Enßlin *et al.* 2011).

Ex situ collections of living plants and viable plant material have the potential to provide a vital insurance policy against extinction in the wild whilst also allowing for the distribution of plant resources, without depleting wild populations, to support scientific research and species' in situ restoration programmes (Guerrant *et al*. 2004; Hurka *et al.* 2004; Li & Pritchard 2009). Opportunities for maintaining diverse ex situ collections of living

plants and plant material are generally more diverse and economic than those available for threatened animal species, particularly as the majority of plant species produce orthodox seeds that maintain long-term viability under conditions of low temperature and low humidity storage (Li & Pritchard 2009). Orchards, arboretums, and botanical gardens maintain living collections of wild plant species. Banked seed accessions, in vitro stored tissue, and embryo cryopreservation collections maintain viable propagation material of wild provenance. Collections of non-viable plant material, such as preserved herbarium specimens and extracted genomic DNA, serve primarily to document plant diversity (Rice 2006; Negri & Tiranti 2010; Thorman *et al.* 2006).

1.4.2 GSPC Targets for Ex situ Conservation: Aims

Target 8 of the 2011 updated GSPC sets out targets for 75% of the worlds threatened species to be held in viable or living ex situ collections by 2020, with 20% of collections available for species in situ recovery and restoration programmes (SCBD 2011). Globally, living plant collections maintained by botanic gardens and seed accessions maintained by seed banks represent the most accessible and greatest capacity ex situ plant conservation resources available to support progression towards GSPC Target 8 outcomes (Harris *et al.* 2009; Sharrock *et al.* 2010).

1.4.2 GSPC Targets for Ex situ Conservation: Progression

The expansion of ex situ plant conservation facilities is one of the noted successes of the GSPC and estimates place 170,000 plant species in ex situ collections with 10,000 of these recognised to be globally threatened (GSPC 2014). The Millennium Seed Bank project, led by RBG Kew involving some 60 international partners, is a notable example of the

enhanced capacity and international collaboration that has emerged within ex situ conservation over the course of the GSPC (Jackson & Kennedy 2009). The ENSCONET (European Native Seed Conservation Network) database lists more than 48,000 seed bank accessions held across its partner countries, representing 52% of Europe's threatened flora (SCBD 2014). In China, the Chinese Academy of Sciences (CAS) hosts the largest group of botanic gardens focusing on the ex situ conservation of native plant species, approximately two thirds of Chinese flora is represented within living or seed bank collections with one third of all ex situ collections duplicated in at least one other facility (Huang 2011). Due to such demonstrable global capacity building it is likely that some countries will achieve the first aim of Target 8 (for 75% of the world's threatened species to be conserved ex situ) by 2020, although this target is perhaps unrealistic for mega diverse countries (Sharrock & Wilson 2014). It is unlikely, however, that the applied aims of GSPC Target 8, to mobilise ex situ plant collections in support of in situ restoration, will be met within this time frame (SCBD 2014; Sharrock & Wilson 2014).

1.4.3 Related GSPC Targets: Ecological Restoration

Utilising viable ex situ plant collections as a tool for restoring plant diversity to natural habitats is an implicit action required to achieve the aims of GSPC Target 4 for *'at least 15% of each ecological region or vegetation type secured through effective management or restoration'*. Whilst international progress is likely insufficient to achieve the 15% benchmark by 2020, foundations for progression towards achieving restoration targets within a wider timeframe have begun to be established via the development of international partnerships, increased capacity within ex situ conservation, and showcasing of a small number of successful restoration projects (SCBD 2014). The 'Ecological

Restoration Alliance of Botanic Gardens' facilitates the collaborative sharing of knowledge, expertise, and strategy for the application of ex situ resources to ecological restoration. The Missouri Botanic Garden's 2,500 acre Shaw Nature Reserve provides a model for the active restoration of native plant communities and ecosystems to recover the biodiversity and ecological functioning of previously degraded habitats (SCBD 2014).

1.5 Ex Situ Conservation Genetics

1.5.1 Value of Ex Situ Wild Plant Resources: Education, Research, and Conservation

The value of living (i.e. botanical gardens) and viable (i.e. seed banks) ex situ plant collections as an accessible and extensive resource for botanical education and research has long been recognised (Crane 2004; Harris *et al.* 2009; Sharrock *et al.* 2010). Viable and non-viable collections of wild plant species, such as herbarium specimens and genomic DNA banks, provide an invaluable opportunity to explore and document fine scale intra and inter species variation. Thereby, ex situ conservation has made a significant contribution towards understanding the extent and distribution of global plant diversity (Crane 2004). However, the value of living and viable ex situ plant collections as an integral conservation mechanism for the safeguard and restoration of global plant diversity (as stipulated under target 8 of the GSPC) remains to be empirically validated (Schoen & Brown 2001; Ramantha Rao & Hodgkin 2002; Li *et al*. 2002; Hurka *et al.* 2004; Rice *et al.* 2006; Harris *et al.* 2009)

1.5.2 Genetic Representativeness of Ex situ Plant Collections

It is accepted that, in the short term, living and viable ex situ conserved collections of threatened wild plant species provides some insurance against extinction in the wild (Li & Pritchard 2009). However, it has also been recognised that there are inherent limitations to

the isolation model of ex situ conservation (i.e. maintaining populations of wild plant species in isolation from natural selection pressures and opportunity for gene flow) that compromise aims for the capture, maintenance, and restoration of genetically representative populations of threatened plant species (Falk *et al.* 2001; Ramantha Rao & Hodgkin 2002; Rodgers 2006; Harris *et al.* 2009; Wall 2009). Genetic diversity is one of three levels of biodiversity recommended for conservation by the International Union for the Conservation of Nature (SCBD 2011). Recommendations emerging from the 2009 progression review of GSPC targets include the implementation of a genetic representativeness success measure for ex situ collections, as opposed to simply measuring the number of species held in collections (SCBD 2010).

In general, when compared to in situ populations, living ex situ plant collections are observed to support reduced levels of genetic diversity and high levels of genetic divergence, i.e. a shifting of allele frequencies away from that observed in natural populations (Negri & Tiranti 2010; Enßlin *et al.* 2011; Rucinska & Puchlaski 2011; Lauterbach *et al.* 2012; Brütting *et al.* 2013). An empirical assessment of the representativeness of ex situ seed bank collections of natural genetic variability is currently lacking. Genetic diversity decline as an inherent risk of ex situ wild plant conservation potentially limits the capacity to contribute to the in situ restoration of natural plant diversity, thereby challenging the status of ex situ collections as a valid conservation resource (Falk *et al.* 2001).

1.5.3 Challenges of Ex Situ Conservation: Capturing Natural Genetic Diversity

Representative Sampling of Natural Genetic Variation

The structuring of genetic variation among populations is a typical feature of the fragmented distribution of plant species distributed across naturally fragmented habitats (i.e. where natural barriers such as mountain ranges interrupt gene flow) and species threatened by habitat loss and ecological disturbance. This partitioning of genetic variation among populations represents a significant challenge to ex situ conservation aims of capturing a representative sample of the full range of a species' natural genetic variability (Clarke & Young 2000; Sherwin & Moritz 2000; Falk *et al.* 2001; Vitt & Havens 2004; Rosetto 2006; Thorman *et al.* 2006; Frankham *et al.* 2009; IUCN 2009; Harris *et al.* 2009).

An optimal ex situ sampling strategy will be species specific, informed by an understanding of spatial patterns in the structuring of genetic variation throughout a species' range (Falk *et al*. 2001; Harris *et al.* 2009). However, comprehensive population genetic studies are available for only a minority of threatened wild plant species represented in ex situ collections (Falk *et al.* 2001). Therefore, ex situ sampling strategies are more frequently designed around proxy measures (such as geographic distance) for genetic variation among populations (Falk *et al*. 2001). When interpreted through simple conservation genetic principles, such proxy measures can be used to infer population genetic structure and inform key collection decisions (Falk *et al*. 2001; ENSCONET 2009). For example, determining the number and location of populations to be sampled which will maximise the potential for capturing a genetically representative ex situ collection within the constraints of available resources (Falk *et al.* 2001; ENSCONET 2009).

Seed banking offers the most efficient means for capturing, distributing, and maintaining, genetically representative ex situ collections for the majority of wild plant species (Sharrock *et al*. 2010). As a baseline recommendation, best practice guidelines

suggest a minimum of five populations should be sampled across a species' range to ensure capture of between 67-87% of allelic variation, with the largest populations identified as the priority for collection when resources are limited (Falk & Holsinger 1991; Falk *et al.* 2001; Neel & Cummings 2003). As many as 50 populations may need to be targeted for sampling throughout a species' range to maximise the potential for seed bank accessions to capture 95% of a species' genetic variation, defining the benchmark for a truly representative ex situ collection (Way 2003; Guerrant *et al.* 2004; Rodgers & Montavlo 2004).

Population genetic (or proxy) information can be used retrospectively to assess the representativeness of existing living and viable ex situ collections and to identify collection priorities for genetic diversification of species collections (SCBD 2014b). In reality, considerations such as the availability of resources for diversifying species collections, and a focus of funding targets on the number of species represented ex situ, tend to take precedence over aims to achieve collections that represent the full range of species' genetic variation (Henry 2006).

Representative Sampling of Population Genetic Diversity

Standardised international protocols have been developed to ensure that seed bank collections (accessions) capture a representative sample of the genetic diversity supported within targeted in situ populations (ENSCONET 2009). A genetically representative seed accession is interpreted as a collection capturing at least 95% of the allelic (gene copy) diversity of the wild provenance population; or, more specifically, captures at least one copy of each allele occurring at a frequency of 5% or more within the population (ENSCONET 2009). The widely accepted baseline recommendation for obligate outbreeding species is for 30 plants, evenly distributed throughout a population to be sampled for seed collection

(ENSCONET 2009). Where the breeding system of a species is uncertain, sampling of 50 plants is recommended as best practice to ensure adequate sampling of within population genetic diversity, i.e. inbreeding and selfing is predicted by conservation genetic theory to reduce individual gene diversity thereby partitioning genetic variation among individuals (Sherwin & Moritz 2000; Frankham 2003; ENSCONET 2009).

Where seed accessions are intended to support species recovery and restoration a sampling intensity of 200 plants is the suggested minimum to ensure allele frequencies of the collection are representative of the genetic profile of the natural population (Brown & Marshall 1995; ESCONET 2009). Recommendations for the minimum quantity of seed captured from a target population vary between 2,500 up to 20,000 dependent on the pressures likely to be placed on the accession in terms of distribution, duplication, and propagation for regeneration of collection viability or for contribution to species' in situ restoration programmes (ENSCONET 2009; Wall 2009).

1.5.4 Challenges of Ex Situ Conservation: Maintaining Natural Genetic Diversity

Maintaining wild plant populations in isolation from natural environmental processes, as a living or viable ex situ collection, will inevitably result in declining viability over time (Falk *et al.* 2001; Ramantha Rao & Hodgkin 2002; Wall 2009). Regeneration (i.e. a reproduction cycle) of collections is a common ex situ practice employed as a measure to restore collection viability (and population size) without necessitating exploitation of natural resources (Walter 2005; RBG, Kew 2014). However, the practice of regeneration exposes ex situ collections to artificial selection factors and to the stochastic genetic sampling effects of genetic drift and founder effect that drive genetic diversity decline within small and isolated

populations (Falk *et al.* 2001; Schoen & Brown 2001; Wall 2009; Negri & Tiranti 2010; Enßlin *et al*. 2011 Lauterbach *et al.* 2011; Rucinska & Puchlaski 2011; Brütting *et al.* 2013).

The mechanism of ex situ conservation utilised will have a significant impact on the extent to which maintenance of collection viability relies on regeneration (Sharrock *et al*. 2010). For example, living botanic garden collections are typically many generations removed from the wild source population (in particular annual and short lived species) whilst seed bank accessions are likely to be no more than one generation removed from the wild provenance population (Sharrock *et al.* 2010). Regeneration of seed bank accessions to rejuvenate collections is recommended by best practice guidelines to be applied when collection viability drops below an accepted threshold, typically 85% (Wall 2012; RBG, Kew 2014). In practice, for the majority of species held in seed bank collections regeneration of collections has not been required, even after several decades, as storage conditions are calibrated to minimise the loss of viability over time (personal communication, Kate Hardwick (RBG Kew,) 4th March 2014).

Maintenance of wild plant species' genetic resources as viable seed accessions within seed banks is facilitated by banking of large, genetically diverse collections, typically 1000's to 10's of 1000's of seeds (RBG, Kew 2014; Wall 2012). In recognition of the enhanced conservation value of seed banking, in comparison to living collections, 275 botanic gardens, in 66 countries, have developed resources for seed banking over the last few decades (Sharrock *et al.* 2010; SCBD 2014b).

1.5.5 Challenges of Ex situ Conservation: Restoring Natural Genetic Diversity

The value of seed banking for the conservation of global plant diversity is contingent upon ex situ practices successfully facilitating the in situ restoration of natural genetic diversity captured and maintained within stored seed accessions (Guerrant *et al.* 2004; Menges et al. 2004; ENSCONET 2009; Harris *et al.* 2009). A productive area of ex situ plant conservation research is therefore the development of species specific germination protocols, in particular breaking dormancy mechanisms that inhibit germination of viable seed (RBG Kew 2014). In theory, maximising germination percentages for viable ex situ banked seed will minimise the risk of genetic diversity decline (i.e. the chance loss of rare alleles) and allelic frequency shifts (i.e. genetic differentiation) in the process of collection regeneration for the propagation of viable plant material (i.e. transplants) suitable for in situ restoration (Meyer & Monsen 1992; Kaye *et al*. 2003; Rodgers 2006).

In general, laboratory germination trials, that are the focus of ex situ conservation research, correlate poorly with field and/or nursery germination trials for the same species and frequently for the same seed accession (Cambell & Sorensen 1984). Laboratory germination trails are primarily designed to test seed viability (and therefore record germination at the point of cotyledon or root emergence) whilst field/nursery germination trials are primarily designed to test fitness to the propagation environment (and therefore record germination at the point of leaf emergence. Ex situ regeneration of banked seed to propagate seedlings for in situ restoration has been observed to result in a high rate of attrition of viable seed, risking a decline in genetic representativeness. (Cambell & Sorenson 1984; Meyer & Monsen 1992; Brown & Briggs 1991).

Whilst population genetic studies of in situ restoration (supported by ex situ propagated transplants) are few, the available literature indicates a significant risk of inbreeding and population genetic structuring, i.e. partitioning of genetic variation among wild provenance and restored populations (Vilas *et al.* 2005; Lloyd *et al.* 2012; Fant *et al*. 2013). Reintroduced populations of *Cirsium pitcher* were observed to have significantly higher inbreeding coefficients than natural populations, attributed to genetic sub-structuring (i.e. the Wahlund effect) and small population size of the founder population (Fant *et al.* 2013). This excess of homozygosity was observed despite the use of multiple genetic sources for the founder population that resulted in greater allelic richness than observed in local wild populations (Fant *et al.* 2013). Vilas *et al.* (2005) also observed high rates of inbreeding within restored populations of a threatened plant species (*Silene littoria*); poor success of this restoration was attributed to the homozygosity excess. Li *et al.* (2005) observed that geographic recovery of *Metasequoia glyptostrobides* in China was not correlated with genetic recovery, whilst genetic diversity of restored populations was equivalent to in situ populations significant population genetic structure was observed among in situ and restored populations. Lloyd *et al.* (2012) also observed significant inbreeding (homozygosity excess) within restored populations of a threatened plant species (*Vallisneria americana*) and genetic divergence from in situ wild populations, attributed to small founder population size. Alternatively, Ritchie & Krauss (2012) achieved successful genetic management of restored populations of *Banksia attenuate* by using diverse local provenance seeds as the founding population and supporting pollinator services to facilitate extensive gene flow with natural populations. Resulting restored populations displayed high levels of allelic diversity and heterozygosity and an absence of population genetic structure with natural populations.

The measurable outcome of success of in situ restoration is the long-term self-perpetuation of restored populations, with further measures of success being population expansion and ultimately dispersal to establish satellite populations (Meyer & Monsen 1992; Menges 2008). Whilst long-term monitoring of threatened plant species restoration actions is limited, a recent comprehensive meta-review of available data found evidence of self-perpetuation in only a minority of studies (Godefroid *et al.* 2011). Knowledge of the population genetic structure of target species and incorporation of simple conservation genetic principles, such as sourcing transplants and/or propagation material (i.e. seed) from large and genetically diverse provenance populations, have been clearly demonstrated as key features of successful (in the long-term) restoration plans (Godefroid *et al.* 2011). The omission of conservation genetic principles as a general feature of failed plant species restoration projects provides a clear demonstration of the difficulties of translating conservation genetic principles into successful conservation management of threatened species (Godefroid *et al.* 2011; Frankham 2003).

1.6 Capture, Maintenance and Restoration of Genetic Diversity: A Molecular Genetic Study

1.6.1 *Anemone pulsatilla* L*:* A Model Species for Research

Anemone pulsatilla L. (*Pulsatilla vulgaris* Mill.) has been selected as a suitable model for a molecular genetic study exploring the ex situ conservation challenges of capturing, maintaining, and restoring the natural genetic diversity of a threatened native UK species. *A. pulsatilla* occupies a restricted UK distribution and it is therefore feasible to sample all extant populations and conduct a study of extant population genetic structure. *A. pulsatilla* therefore provides a valuable opportunity to explore the impact of fragmentation on the

distribution of genetic variation across a species' range. *A. pulsatilla* has also been the subject of a number of ex situ conservation actions in recent years including the establishment of seed bank accessions, ex situ regeneration of seed bank accessions, and the mobilisation of ex situ resources for contribution to in situ restoration programmes. *Anemone pulsatilla* therefore provides a unique opportunity to explore the effectiveness of current ex situ conservation measures in supporting the ex situ safeguarding and in situ restoration of the natural genetic diversity of a vulnerable (IUCN threat criteria) UK species (Walker 2011a).

1.6.2 *Anemone pulsatilla* L: A Species in Decline

The pasqueflower (*A. pulsatilla*) is a perennial rhizomatous herbaceous species of unimproved, botanically rich, chalk and limestone (calcareous) grassland. The species occupies a highly fragmented European range (figure 1), the northern limit of distribution falling in Scandinavia and the southern limit in Bordeaux, France (Wells & Barling 1971; Henson *et al.* 2005; Walker & Pinches 2011). The species has traditionally been associated with Easter, being one of the first grassland species to flower in spring, hence the common English name derived from Paschal (Easter). The localised distribution and attractive early and prolific (under good management) flowering of *A. pulsatilla* has contributed to the species status as a flagship for grassland conservation in the UK.

Extinct in Finland and the Netherlands (and possibly Poland at its eastern range limit) *A. pulsatilla* is in decline throughout its range and is classified as a European near threatened species under IUCN threat criteria (Henson 2005; IUCN Criterion A2ac; Cheffings & Farrell, 2005; Walker 2011a; Walker & Pinches 2011; Schweizer & Hasinger 2014). Decline in the availability of species rich chalk and limestone grassland, due to extensive

land use change (i.e. quarrying, ploughing for arable, agricultural improvement) and the abandonment of traditional grassland management practices, have been cited as key deterministic factors driving the species' European decline (Henson *et al.* 2005; Walker & Pinches 2011; Schweizer & Hasinger 2014).

In the UK, as across much of the species' European range, *A. pulsatilla* extant distribution (figure 2) represents the fragmentation of a former far more expansive and continuous range (figure 3). Fragmentation has been particularly high in East Gloucestershire (an historical centre of population density for the species), South Lincolnshire, and Berkshire, representing the western, northern, and southern regions of the species' range respectively (Walker 2011a). The centre of population density for *A. pulsatilla* is located across the counties of Bedfordshire, Hertfordshire and Cambridgeshire in the eastern region of the species' range where four of the five largest extant populations are located.

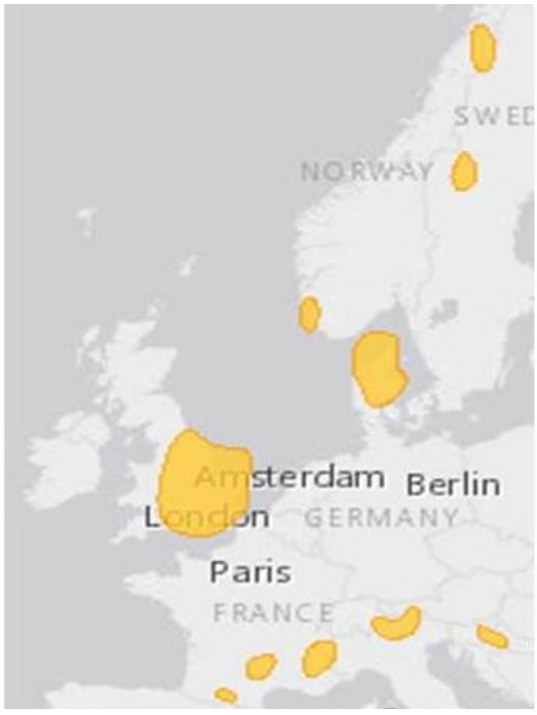


Figure 1. *Anemone pulsatilla* L. extant European distribution, figure taken from IUCN 2014.

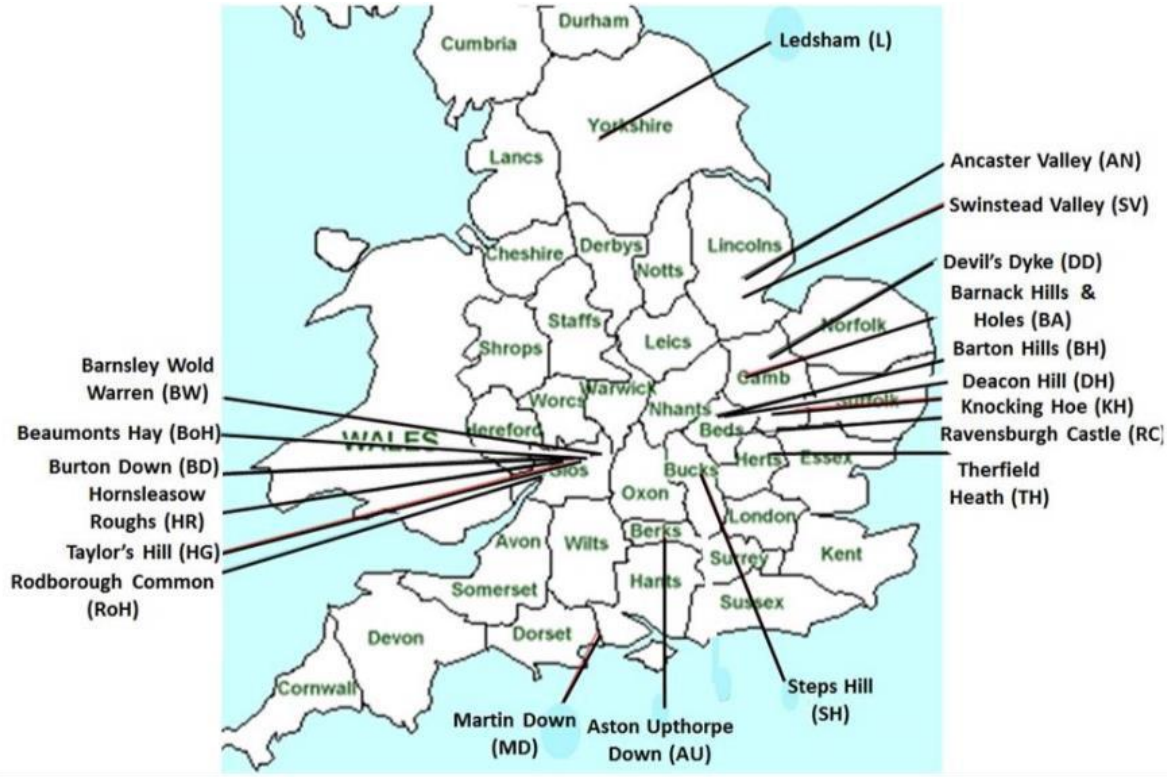


Figure 2. The distribution of extant UK populations of *Anemone pulsatilla* (adapted from Walker 2011a) inclusive of the putative introduction (MD) that falls outside of the species' historical UK range, and the populations AN and AU which have been subject to restoration intervention in the form of augmentation (i.e. the introduction of ex situ cultivated transplants), labels refer to population name (code).

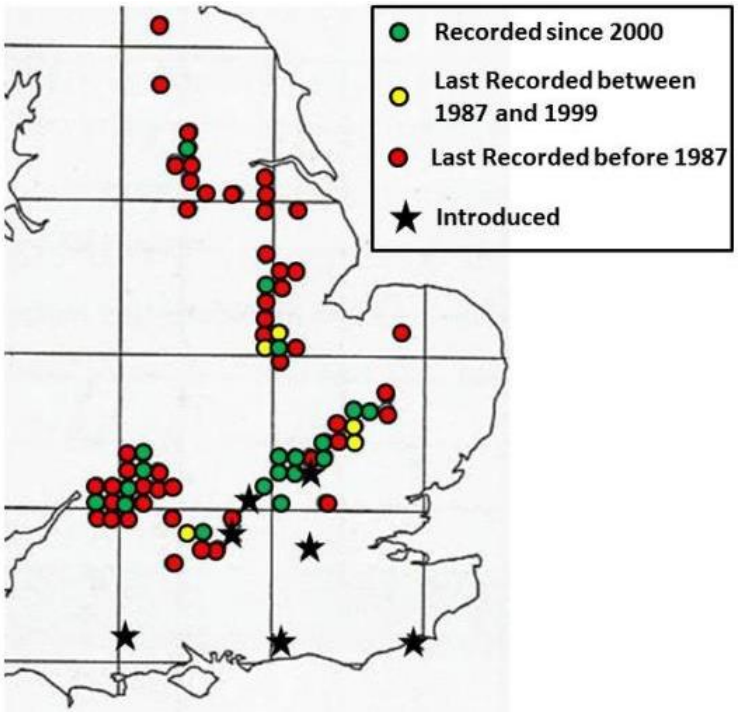


Figure 3. The decline in hectared distribution of *Anemone pulsatilla* over the latter half of the 20th Century (adapted from Walker 2011a).

The ecology and life history of *A. pulsatilla* account, in part, for the species' vulnerability to declining quality and fragmentation of available habitat (Walker 2011a). Typically, UK populations of *A. pulsatilla* occur on south or south west facing slopes of dry pasture, ancient earth works, and old quarry workings where insolation is high and the shallow, nutrient poor, soils restrict the growth of competitive species (Wells & Barling 1971). As is a typical feature of perennial plant species confined to low nutrient calcareous grassland habitat, *A. pulsatilla* is a poor competitor and coloniser (Piqueray *et al*. 2013). Competitive ability of *A. pulsatilla* is limited by the species' vulnerability to competitive exclusion by more vigorous species such as the coarse grasses that dominate calcareous grassland communities in the absence of appropriate, low intensity, management regimes such as mowing or typically grazing (Wells & Barling 1971; Walker & Pinches 2011). Colonisation ability of *A. pulsatilla* is limited by short distance seed dispersal, a transient short-lived seed bank, and low recruitment from seed observed in natural populations (Wells & Barling 1971; Thompson *et al.* 1997; Bisteau *et al.* 2005; Piqueray *et al.* 2013). Poor recruitment from seed has been observed for *A. pulsatilla* in both natural and ex situ propagation environments, despite high seed viability and an absence of seed dormancy observed in standardised laboratory germination trials (Walker & Pinches 2011; Piqueray *et al.* 2013). Poor correlation between germination rates observed in the laboratory viability testing and survival rates observed in nursery and field settings is a typical feature of many wildflower species (Meyer & Monsen 1992).

The most recent UK national assessment of *A. pulsatilla* recorded 19 extant UK populations (inclusive of a putative casual introduction site Martin Down MD), a significant decline from the 33 populations recorded in the previous national survey conducted in 1968

(Wells & Barling 1971; Walker & Pinches 2011). The 19 extant sites identified by Walker and Pinches (2011) for *A. pulsatilla* in the UK (Table 1.) are considered as distinct populations due to the discrete distribution of individuals within these sites and the likely absence of gene flow among these sites. Seed dispersal distance is low for *A. pulsatilla* (c. 20cm to 300m), and foraging radius of pollinators (bees and bumblebees) is thought to be too low to connect extant populations through pollen exchange (Steffan-Dewenter & Kuhn 2003, Leslie 2004, Osbourne *et al.* 2008, Walker 2011, Wells & Barling 1977).

A decline in quality of available calcareous grassland habitat, characterised by an increase in the cover of coarse grasses (i.e. *Bromus erecta* and *Brachypodium pinnatum*), is cited as the key deterministic factor driving population decline (Walker & Pinches 2011). The widespread abandonment of traditional grassland management practices (i.e. low density cattle/sheep grazing) is understood to be largely responsible for the decline in availability of good condition chalk and limestone grassland since 1968 (Walker & Pinches 2011).

Table 1 Details of extant Anemone pulsatilla populations in the UK, [1]from Walker (2011), [2] six native plants and 22 introduced transplants were recorded by Walker (2011); [3]one plant remains, [4]site falls outside of the historical UK range of *A. pulsatilla* and is thought to be an introduction.

Site No.	Site Name	Site Code	Location	Pop. Size[1]
1	Therfield Heath	TH	Hertfordshire	>10000
2	Aston Upthorpe Down	AU	Berkshire	11-100
3	Steps Hill	SH	Buckinghamshire	11-100
4	Devil's Dyke	DD	Cambridgeshire	101-1000
5	Barton Hills	BH	Bedfordshire	1001-10000
6	Deacon Hill[2]	DH	Bedfordshire	1-10
7	Knocking Hoe	KH	Bedfordshire	1001-10000
8	Ravensburgh Castle	RC	Bedfordshire	11-100
9	Barnack Hills & Holes	BA	Cambridgeshire	>10000
10	Barnsley Wold Warren	BW	East Gloucestershire	>10000
11	Beaumonts Hay	BoH	East Gloucestershire	1-10
12	Bourton Down	BD	East Gloucestershire	101-1000
13	Hornsleasow Roughs	HR	East Gloucestershire	101-1000
14	Taylor's Hill	HG	East Gloucestershire	101-1000
15	Rodborough Common[2]	RoC	West Gloucestershire	11-100
16	Ancaster Valley[3]	AN	South Lincolnshire	11-100
17	Swinstead Valley	SV	South Lincolnshire	1-10
18	Ledsham	L	Mid-West Yorkshire	1-10[4]
19	Martin Down[5]	MD	Dorset/Hampshire	1-10

Whilst the majority of extant UK populations are small (<100 individuals), and/or declining, a minority of populations have experienced a significant increase in population size since 1968 with three population sites recorded as supporting >10,000 individuals (flowers) in 2011, see table 1 (Walker & Pinches 2011). These recent population expansions, confined predominately to the eastern region of the species' extant UK range (i.e. the centre of population density), have resulted in a 258% increase in total UK population size (quantified by number of flowers) since 1968 (Walker & Pinches 2011). Population size (i.e. extent of flowering) increase can largely be attributed to the reinstatement of appropriate management regimes and the recovery of good condition, i.e. a reduction in coarse grass and increased floristic diversity, at select *A. pulsatilla* sites (CEH 2007; Walker 2011b).

Key concerns regarding the conservation status of *A. pulsatilla* in the UK include the isolation (in terms of pollinator assisted gene flow) assumed for all populations and the geographical bias in distribution of individuals throughout the species' extant range (Walker 2011a). Five population sites are recorded to support >99% of the total UK population, with four of these sites falling within the eastern region of the species' extant UK distribution (Walker 2011a). The largest UK popualtion, Barnsley Warren (BW), is located within an historical centre of popualtion density for the species in the western region of the speces extant range (Walker & Pinches 2011). With the majority of fragmented *A. pulsatilla* popualtions small (<100 individuals) and isolated, there exists a high risk of genetic diversity decline (and potentially the chance loss of adaptive diversity) as a consequence of genetic drift. There also existst a high potential for the emergence of popualtion genetic structure, i.e. the partitioning of genetic varaiton at random among fragmented popualtions, as a consequence of stochastic genetic sampling effets of genetic drift and the founder effect.

The viabiity of the majority of extant UK popualtions of *A. pulsatilla* may therefore be at high risk from declining adaptive genetic diversity and therefore a reduction in fitness to the local envronment and loss of evolutionary potetnial. As a tetraploid species (four as opposed to two allele copies at each gene locus) fragmented *A. pulsatilla* populations may experience a lower risk than diploid species of the chance loss of genetic diversity though the sampling effects of genetic drift and founder effect.

In support of *A. puslatilla* conservation in the UK, Walker & Pinches (2011) define a number of research priorities for this species. These include: (a) an assessment of the distribution of genetic variation among UK populations; (b) an understanding of the role of inbreeding and genetic drift in influencing population fitness; and, (c) an investigation of ecological factors (such as community interactions, dispersal mechanisms, and regeneration niche) that limit species competitive and colonisation ability. The National Trust (NT) and RBG, Kew, are leading the response to these recommendations through establishment of a research and species recovery project entitled 'Towards the Landscape Scale Restoration of *A*. *pulsatilla*'. This project also addresses recommendations of two recent statutory reports that assess the status of UK biodiversity; "Making Space for Nature: A review of England's Wildlife Sites and Ecological Network", chaired by Professor Sir John Lawton CBE FRS and submitted to DEFRA in 2010, plus "Biodiversity 2020: A strategy for England's wildlife and ecosystem services" by DEFRA in 2011.

1.6.3 *Anemone pulsatilla* L.: In situ Conservation Action

A clear finding to emerge from the Walker & Pinches (2011) assessment of *A. pulsatilla* distribution in the UK is that population persistence is dependent upon the active and appropriate management of unimproved chalk and limestone grassland sites. Six

grassland sites supporting stable or increasing *A. pulsatilla* populations have been recorded to be in good condition, i.e. high floristic diversity with low coverage of coarse grasses, due to the establishment of low intensity grazing regimes (Walker 2011b). A further six sites have been recorded as 'recovering good condition' due to the reinstatement of appropriate grazing regimes and, in some cases, habitat restoration intervention to control wide spread scrub encroachment. The introduction of subsidised environmental stewardship schemes have supported much of this improvement in the management of previously neglected *A. pulsatilla* sites (Walker 2011b). Remaining sites supporting extant *A. pulsatilla* populations are recorded as being in poor condition, a result of transition to coarse grass dominated vegetation communities and the encroachment of scrub through management neglect (Walker 2011b). Populations of *A. pulsatilla* recorded at these sites of poor habitat condition are recorded as supporting <100 individuals (flowers) and are assessed to be vulnerable to extinction without conservation management intervention (Walker 2011b). As a long lived species, it is possible that populations of *A. pulsatilla* persist at a further five sites in a vegetative state and may recover in the event of habitat restoration through the reinstatement of appropriate management (Walker 2011b).

In situ management priorities for extant *A. pulsatilla* populations are to secure the long-term appropriate management of sites in good and improving habitat condition and to identify habitat restoration options for sites in poor condition. Sites supporting <100 individuals may require restoration intervention (i.e. the introduction of translocated or ex situ propagated (wild provenance) transplants) to reverse the trend of population decline and to enhance population viability through the introduction of adaptive genetic diversity. In the long-term, population reintroduction (using representative ex situ plant material) as a

strategy to recover landscape connectivity, thereby allowing for the distribution of genetic variation throughout the species' UK range, is likely to be critical to securing the long-term viability of the UK distribution of *A pulsatilla*.

1.6.4 *Anemone pulsatilla* L.: Ex situ Conservation Action

As a species vulnerable to extinction in the UK, *A. pulsatilla* is the subject of an ex situ conservation strategy aimed at safeguarding natural genetic variability and supporting in situ restoration. Much of the ex situ conservation of *A. pulsatilla* has been coordinated by the Millennium Seed Bank (MSB) based at Wakehurst Place (RBG WP), Jodrell Laboratory (RBG Kew), and the National Trust (NT). Actions include the capture and maintenance of wild provenance seed accessions (MSB), see table 2 for summary. Also, the ex situ propagation of wild provenance seed (regenerated from MSB seed accessions) to establish F1 ex situ living collections marinated at RBG WP and Jodrell Laboratory (RBG Kew), see table 3 for a summary. An F2 ex situ propagated living collection (regenerated from a wild provenance F1 ex situ living collection) is also maintained a RGB WP.

Ex situ regeneration of *A. pulsatilla* wild provenance seed has been applied to provide transplants for in situ restoration actions over the last two decades, with variable establishment success (see table 4 for summary). Observations of self-perpetuation have been recorded for formal restoration trials at Hartslock (HA) and Aston Upthorpe Down (AU), established from introduced transplants one generation removed from the wild provenance population (Warden 2001 unpublished MSc Thesis; Warden 2012). Establishment success (although without signs of self-perpetuation) has also been observed for augmentation of Ancaster Valley (AN) population site where survival of introduced transplants stands at approximately 30%. Introduced, ex situ regenerated, transplants are

understood to be of AN wild provenance. However, the ex situ regeneration practices applied in the propagation of transplants has not been recorded (genetic provenance therefore cannot be fully verified). Establishment has been unsuccessful for other A. *pulsatilla* restoration attempts.

It can be observed from table 3 that ex situ regeneration of *A. pulsatilla* wild provenance seed has achieved consistently low (i.e. <50%) germination rates. The highest germination percentage achieved across RBG WP and Jodrell Laboratory (RBG Kew) propagation trials for Barnack Hills and Holes (BA) wild provenance seed (sourced from a MSB seed accession collected in 1999) is 30%. However, 90% seed viability was recorded in the most recent MSB laboratory seed viability test carried out in 2012 (seed information database, RBG WP). Therefore, ex situ regeneration of *A. pulsatilla* carries a high risk of attrition of viable seed and, as a consequence, a high risk of genetic diversity decline via stochastic sampling effects. This risk, which is inherent to the ex situ conservation processes of regenerating seedlings from banked seed, compromises the potential for restoring populations of *A. pulsatilla* with adequate adaptive genetic diversity.

Table 2 Ex situ seed bank accessions for *A. pulsatilla* in the UK, [1]taken from Walker (2011a), [2]MSB records in 2013, [3]Low humidity and low temperature conditions to allow long-term maintenance of collection viability, [4]awaiting processing before storage.

Provenance (wild) Pop. Code (Location)	Provenance (wild) Pop. Size[1]	Collection Year	Collection Type	Collection Size[2]	Facility
BA (Northants)	> 10000	1999	Seed	15819	Millennium Seed Bank, RBG Kew: In Storage[3] Accession Number 134886[4]
SH (Bucks)	10-100	2013	Seed	616	Millennium Seed Bank, RBG Kew: Drying Room[5]

Table 3 Ex situ regenerated (living) populations of *A. pulsatilla,* [1]number of generations removed from wild provenance population, [2]% of seed lot which survived to potential restoration transplant size c. 4 months after germination (i.e. juvenile plant), [3]SH seed collected in 2013, [4]averaged across three germination treatments with the most successful treatment resulting in 54% conversion.

Provenance (wild) Pop.	Regeneration Cycle[1]	Origin of Regenerated (Parent) Seed	Regeneration Facility	Regeneration Year	Survivorship %[2]
BA	First	MSB , RBG Kew Accession:134886	Wakehurst Place, RBG WP	2011	n/a
BA	First	MSB , RBG Kew Accession:134886	Wakehurst Place, RBG WP	2012	16
BA	First	MSB , RBG Kew Accession:134886	Wakehurst Place, RBG WP	2013	17
BA	First	MSB , RBG Kew Accession:134886	Wakehurst Place, RBG WP	2014	30
BA	Second	Wakehurst Place 2012 Regeneration	Wakehurst Place, RBG WP	2014	28[4]
BA	First	MSB , RBG Kew Accession:134886	Jodrell Lab., RBG WP	2013	10
SH	First	SH[3]	Jodrell Lab., RBG WP	2013	10

Table 4 Restoration actions for *Anemone pulsatilla,* [1]provenance of ex situ transplants and number of generations regenerated ex situ prior to introduction to AN are not verified, [2,3]restoration trial coordinated by Katy Warden (RBG Kew), [4]transplant refers to introduction to restoration site of a juvenile or mature plant propagated ex situ, [5]regeneration refers to introduction to restoration site of viable seed, [6]taken from Walker (2011a) with the exception of Ashridge Estate, [7]Sarah Barlow, RBG Kew personal communication

Provenance (wild) Pop.	Restoration Site	Restored Population Code	Restoration Action	Restoration Material	Survivorship %[6]	Restoration Year
AN	AN	n/a	Augmentation[1]	Transplant[4]	32	1992-2002
AN	Copper Hill, Lincs.	n/a	Reintroduction	Transplant	0	1994-1996
BH	Hartslock Reserve (Ox.)	HA	Introduction[2]	Transplant	76	1998
BA	Southorpe Paddock (North Hants)	n/a	Reintroduction	Transplant	0	1999
AU	AU	AU_au	Augmentation[3]	Transplant	58	1999
BA	L	n/a	Augmentation	Transplant	0	2000
BA	Ashridge Estate (Beds)	AR	Reintroduction	Regeneration (seed)[5]	13[7]	2013

1.6.5 *Anemone pulsatilla* L.: Population Genetic Study of In situ Populations

The extensive fragmentation of the UK range of *A. pulsatilla* places the species at high risk of the emergence of population genetic structure. Conservation genetic theory predicts that a correlation between geographic and genetic distance will emerge among populations as an artefact of the stochastic genetic sampling effects that underlie the development of population genetic structure (Frankham *et al.* 2013; Luikart *et al.* 2013). Aspects of the species' biology that act to limit gene flow and population expansion (such as low distance seed dispersal and low occurrence of regeneration from seed) act to heighten the risk that genetic variation will become partitioned among in situ populations.

The small (<100 individuals) and/or declining population size and reproductive isolation that typifies the majority of *A. pulsatilla* in situ UK populations places these populations at a high risk of declining genetic diversity, a result of the chance loss of rare alleles via stochastic genetic sampling effects (Frankham *et al.* 2002). Research to understand the role of stochastic genetic sampling effects in shaping population genetic structure of *A. pulsatilla* in the UK, with the aim to inform long-term species restoration and recovery plans, is a key recommendation to emerge from Walker & Pinches (2011) assessment of *A. pulsatilla* UK conservation status. A study of neutral genetic variability (free from the filtering effects of natural selection) is most appropriate for understanding the impact of fragmentation on population genetic processes (Sherwin & Moritz 2000; Vilas *et al.* 2005; Frankham 2010; Ouberg *et al.* 2010).. A neutral marker study of genetic structure among 11 German populations of *A. pulsatilla* recorded a correlation of genetic and geographic distance among populations (Henson *et al*. 2005).

A limited UK population genetic study of *A. pulsatilla* was conducted by Bailey (1996) sampling five neutral gene loci for five in situ populations. The sampling intensity of this study (i.e. number of individuals, populations, and microsatellite loci) falls below that which is understood to reliably identify the impact of stochastic genetic sampling effects (such as founder effect and genetic drift) on the emergence of population genetic structure (Luikart *et al* 1998). However, some indication of among population differentiation can be inferred from this study, providing a sufficient empirical basis to justify further research.

A comprehensive study of *A. pulsatilla* population genetic structure was initiated at Jodrell Laboratory, RBG Kew in 2012 (Pike *et al.* 2014). Ten microsatellite markers were developed for this study, thereby meeting evidenced based recommendations for the population genetic detail required to detect the influence of stochastic sampling effects (such as founder effect, inbreeding and genetic drift) on population genetic structure (Luikart *et al*. 1998). Initial exploratory population genetic analyses of microsatellite data for 15 in situ populations, performed using Polysat package (in R v3.0.2 console), revealed a broad correlation between geographic and genetic distance among in situ *A. pulsatilla* populations distributed along an east to west axis (Pike *et al.* 2014). A proportion of the microsatellite genotypes representing the Ancaster Valley (AN) population were shown to be strongly genetically diverged from the microsatellite genotypes of all other UK populations (Pike *et al.* 2014). Ancaster Valley (AN) is known to have been the subject of a casual augmentation, i.e. a restoration intervention aimed at expanding population size and/or introducing appropriate genetic diversity, perhaps accounting for the sub structuring of genetic variation observed among sampled AN genotypes (Godefroid *et al.* 2011; Walker 2011b). Further research is required to: (a) include additional in situ populations within the

on-going population genetic study; and, (b) to apply statistical modelling approaches (such as cluster analysis) to understand the partitioning of genetic variation among extant UK populations.

1.6.6 *Anemone pulsatilla* L.: Population Genetic Study of Ex situ Populations

RBG Edinburgh's assessment of ex situ conserved resources for UK threatened species places *A. pulsatilla* natural genetic variability at high risk of under-representation within existing ex situ collections (Neaves 2014). High risk status for *A. pulsatilla* is justified by the small proportion of UK populations represented within existing ex situ collections and the high potential for the structuring of genetic variation among natural UK populations (Neaves 2014). Current recommendations are therefore for all natural UK populations of *A. pulsatilla* to be represented within ex situ collections (Neaves 2014). An understanding of the partitioning of genetic variation across the UK distribution of *A. pulsatilla* will allow for the prioritisation of collection decisions to diversify existing ex situ resources.

The ultimate value of ex situ conserved resources for threatened UK plant species is the potential to support restoration and recovery of species' geographic and genetic structure within the natural environment. Therefore, the status of *A. pulsatilla* ex situ conserved resources as a valid in situ restoration tool is contingent upon the capture and maintenance of representative natural genetic diversity. Existing living ex situ collections of *A. pulsatilla* propagated and maintained at Wakehurst Place (RBG WP) and Jodrell Laboratory (RBG Kew) are at present excluded from in situ restoration programmes on the basis that survivorship percentages for these populations fall below 50% of viable seed (personal communication, Vicky Foden (RBG Kew), 3rd April 2014). At less than 50%

survivorship ex situ regenerated living collections for *A. pulsatilla* are assumed to carry a high risk of failing to maintain a representative sample of the natural genetic diversity.

The 50% survivorship threshold employed by RBG WP as a baseline for inclusion of ex situ regenerated living collections with in situ restoration programmes is a generic figure without robust empirical basis (personal communication, Vicky Foden (RBG Kew), 3rd April 2014). Aspects of *A. pulsatilla* biology and life-history (i.e. as a tetraploid predominately outbreeding species) may potentially allow for maintenance of representative genetic diversity under high selection pressure. Therefore, 50% survivorship baseline for inclusion within in situ restoration programmes may be overly conservative, prohibitively so for a species such as *A. pulsatilla* that typically experiences low recruitment from seed in both in situ and ex situ environments.

A number of casual and formal trial restoration interventions have been implemented for *A. pulsatilla* over recent years with variable establishment success, see table 4 for details. The populations at Martin Down (MD) and Ancaster Valley (AN) have been subject to casual (i.e. incomplete record of restoration practices) restoration interventions and have become established within the species' in situ UK range, as such these populations are included within the current *A. pulsatilla* in situ population genetic study. Hartslock (HA), Aston Upthorpe Down (AU), and Ashridge Estate (AR) have been subject to formal restoration trial interventions within the species' natural UK range. Formal refers to the fact that genetic provenance of introduced transplants/propagation material, and the ex situ regeneration and in situ restoration practices applied, are reliably recorded and follow best practice recommendations. Including ex situ regenerated and restoration

trial populations within a population genetic study of *A. pulsatilla* provides an opportunity to contribute to the under researched area of ex situ conservation genetics.

2 Thesis Aims and Objectives

2.1 Research Questions

This research study aims to provide an evidence base for the development of complimentary ex situ and in situ gene conservation strategies for the vulnerable UK plant species *A. pulsatilla.* Recommendations emerging from this study for the safeguard and restoration of the species' natural genetic diversity will be incorporated into the wider project 'Towards the landscape scale restoration of *A. pulsatilla*' led by RBG Kew, and Natural England. Key research questions to be addressed by this study are, as follows:

2.11 Research Question 1:

How has range fragmentation, population decline, and restoration intervention influenced the genetic structure (i.e. among population genetic variation) of *A. pulsatilla* throughout the species' extant UK range?

2.12 Research Question 2:

How well do ex situ conservation measures for *A. pulsatilla* represent the species' natural genetic variability?

2.2 Research Hypotheses

The sampling strategy, methodology, and analytical approached applied in the course of this study are therefore designed to test hypotheses formulated to address the key research questions above, these hypotheses are informed by the principles of conservation genetic theory discussed in previous sections and knowledge of *A. pulsatilla ,* ecology and biology.

2.21 Hypotheses for Research Question 1:

Hypothesis 1(a): The geographical pattern of range fragmentation across the UK range of *A. pulsatilla* will be reflected in the spatial structuring of among population genetic variation, due to random drift of population allelic frequencies in the absence of gene flow among remnant populations.

Hypothesis 1(b): Declining *A. pulsatilla* population size will be associated with declining representation of the species' natural genetic variation, due to the disproportionate influence of random selection factors (such as genetic drift and the bottleneck effect) on the allelic frequencies of small populations isolated from gene flow, i.e. increased risk of the chance loss of low frequency alleles.

Hypothesis 1(c): The introduction of propagules of unverifiable genetic origin to the species' UK range will result in structuring of genetic variation, due to divergence from natural population allelic frequencies.

2.22 Hypothesis for Research Question 2:

Hypothesis 2: Ex situ conservation practices for the establishment and maintenance of accessions can impose high selection pressure on genetic diversity resulting in under-representation of natural genetic variation, due to absence of gene flow and natural selection pressures resulting in drift from natural allelic frequencies.

2.3 Research Approach

The study will aim to sample genotypes (using 10 microsatellite loci) from all extant UK populations of *A. pulsatilla,* inclusive of in situ natural and restoration intervention populations, and all *ex situ* conserved accessions (i.e. seedbank accessions) and *ex situ* regenerated accessions (representing F1 and F2 generations of the parental seedbank accession) from the UK native seed hub (RBG, Wakehurst Place).

Graphical (i.e. Principle Coordinate Analysis (PCA) and Principle Component Analysis (PCoA)) and modelling approaches (i.e. STRUCTURE, and CLUMPP) will be applied for analysis of *A. pulsatilla* population genetic structure, F_{ST} calculations allow for a statistical analysis of population genetic differentiation (i.e. the extent of allelic frequency divergence). Spatial autocorrelation can be applied to test spatial (i.e. geographical) trends in the structuring of population genetic variation across the fragmented UK distribution of *A. pulsatilla.* Estimates of allelic richness (i.e. mean loci polymorphism) can be applied to assess variation in within population genetic diversity.

3 Method

3.1 Population Sampling

Obtaining genetic material from *A. pulsatilla* is achieved by taking small leaf samples from which genomic DNA can be extracted. Extracted DNA (containing both nuclear and plastid genomes) can be utilised immediately for conservation genetic research or stored as a non-viable ex situ collection that can be made available for research at a later date. Sampling in situ and restored populations in the field for leaf material is achieved with greatest success in the early months of the plants emergence from winter senescence, i.e. April to early May, when the large purple flower heads can clearly be observed above the surrounding sward (yet to enter the main grassland growing season). Sampling small, sporadically distributed, populations can be problematic as flowers do not necessarily emerge every year and plants can be difficult to identify from vegetation alone as the finely dissected, low growing, leaf rosettes can be easily overlooked in a tall dense sward, even with thorough searching. Small populations and/or highly dispersed populations therefore risk being excluded or under-represented within a genomic DNA collection for *A. pulsatilla* UK genetic variability.

To obtain a representative sample of a population's genetic diversity, i.e. 95% allelic capture, and ensure reasonable probability of detecting variation in allele frequencies among populations it is recommended that a minimum of 30 individuals be sampled (Luikart *et al.* 1998). A number of *A. pulsatilla* extant in situ populations are thought to number fewer than 100 individuals, for these populations locating 30 or more individuals for leaf sampling is likely to be problematic, particularly for sites in poor condition as tall swards are

likely to inhibit flowering. Five in situ populations (DH, BoH, SV, L, and MD) are thought to have numbered <10 individuals at the time of the Walker & Pinches (2011) survey, these populations risk exclusion from sampling due to difficulty in locating plants for sampling.

Between 2012 and 2013, 15 of the 19 *A. pulsatilla* UK populations recorded by Walker & Pinches (2011) were sampled for leaf material by a RBG Kew staff and a number of volunteers. As part of the current study, a further two in situ populations were visited by the primary investigator (GW) and RBG Kew staff in 2014 with the aim to include additional population's at the western range edge (Gloucestershire) of the species' extant distribution. Plants were only identified at one of these Gloucestershire sites, however. To date, leaf samples have been obtained from 16 in situ populations (table 5), including the casual introduction site (Martin Down) and the casual augmented population Ancaster Valley (AN). Leaf samples collected at the AN site include native and introduced plants, the origin of leaf samples were not recorded at the time of sampling however. As expected, leaf samples from small populations (fewer than 100 individuals) typically numbered <30 individuals. Plants could not be located at three of the in situ populations identified by Walker & Pinches (2011), it is possible that those populations have disappeared from these sites or that plants persist in a vegetative state but failed to flower at the time of sampling (RoH visited in 2014, BoH and DH visited in 2013).

In 2014 three in situ restoration trial populations were sampled for leaf material (table 6). The sampled populations represent three well established restoration trials: (a) Hartslock (HA) introduced population (BH provenance); (b) Aston Upthorpe augmented population (Au_au) where introduced plants (AU provenance) are located in an enclosure separated from the native AU population, and; (c) the Ashridge Estate (AR) introduced

population (BA and SH provenance). Due to the admixed ancestry of AR 60 leaf samples were sampled with the aim to equitably represent SH and BA provenance genotypes with the population. See figure 4 for the distribution of in situ and restoration trial populations of *A. pulsatilla* sampled for the RBG Kew 2012 to 2014 population genetic study.

When conducting a sampling visit to in situ and restoration trial populations an initial survey is conducted to identify population distribution, for smaller populations marker flags can be used to identify individual plants. Sampling is then able to proceed strategically to ensure even coverage of population distribution. As recommended by collection guidelines, donor plants located within in situ and restoration trial populations were sampled at a minimum of 5m apart to facilitate wide and even coverage of the population (ENSCONET 2009). Thus promoted genetically representative collections that exclude duplication through inadvertent sampling of clonally reproduced plants of the same maternal origin. The minimum collection size aimed for was 30 individual plants; however, as explained, small population size and difficulty in identifying *A. pulsatilla* plants in a tall sward (particularly for populations with low occurrence of flowering individuals) limited collection size for many in situ populations.

Between 2013 and 2014 nine ex situ regenerated populations were also sampled for leaf material, populations were maintained in nursery settings in Wakehurst Place (RBG WP) and Jodrell Laboratory RBG Kew (table 7). For ex situ regenerated populations donor plants were individually labelled, an action to allow data on phenotypic traits to be collected at a later date.

To obtain sufficient genetic material for DNA extraction and microsatellite Polymerase Chain Reaction (PCR), approximately thumb size leaf samples were taken from all selected donor plants. The exception to this rule was the sampling of in situ regenerated seedlings at Ashridge Estate (a restoration trial population proceeding by seed addition) where small leaf samples were taken to minimise the risk of influencing the survivorship of sampled individuals. Leaf samples are immediately placed in zip lock sample bags with silica sand to facilitate desiccation of the leaf, aiding subsequent DNA extraction and prolonging sample integrity. Bags are labelled with the date and location (site name) of collection, the species' name, number of collection, and the identity of the collector.

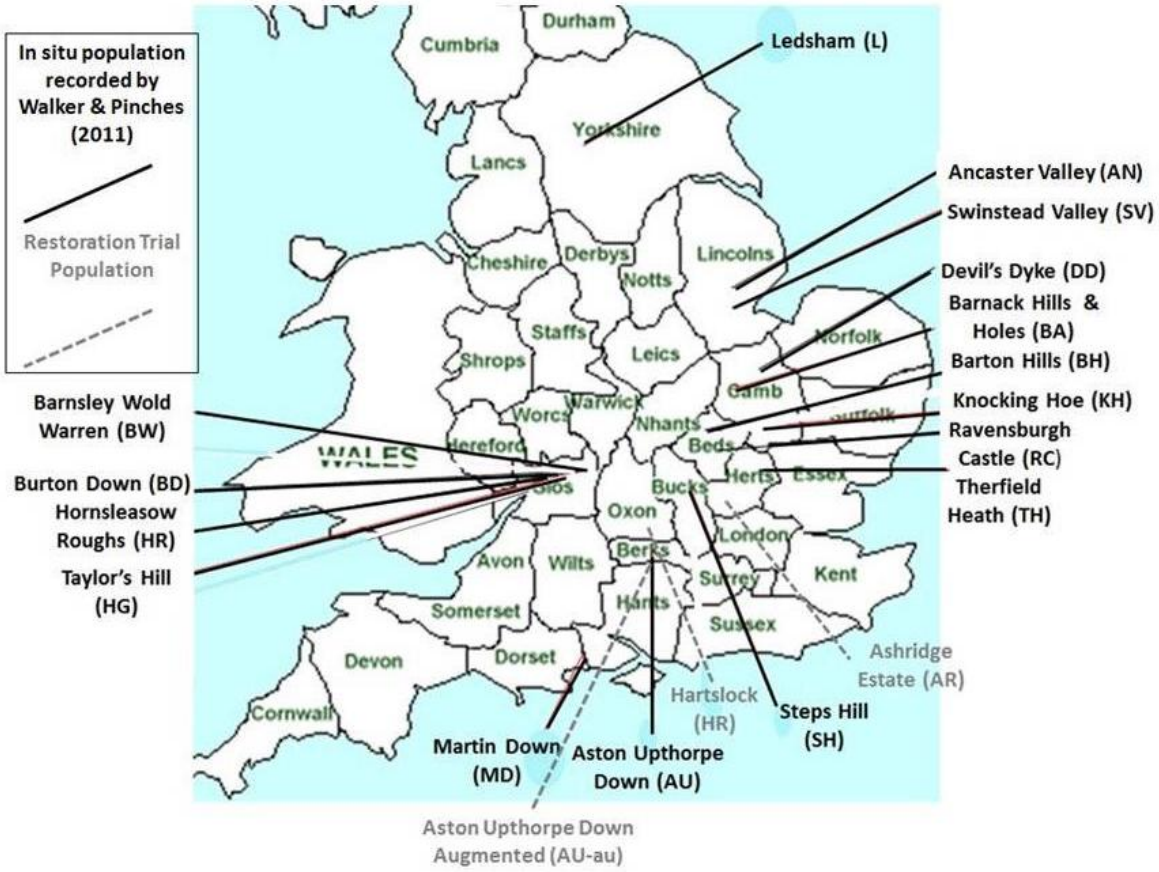


Figure 4. Distribution of *Anemone pulsatilla* L. in situ and restoration trial populations included within a population genetic study, map adapted from Walker (2011a).

Table 5 In situ populations of *Anemone pulsatilla L.* sampled for genetic material for inclusion within a population genetic study conducted at RBG Kew, [1]transplanted individuals were included in the leaf samples taken from AN, therefore a minimum of six samples represent the native population (samples are not labelled to indicate if native or transplant).

Site Code	Pop. Size	Location	Number of Samples	Collector	Collector Affiliation
TH	>10000	Hertfordshire	31	Corinne Arnold; Sarah Barlow; Kevin Walker; Peter Stroh	RBG Kew; Botanical Society of the British Isles (BSBI)
AU	11-100	Berkshire	8	Kathy Warden	U. Oxford Botanic Garden
SH	11-100	Buckinghamshire	12	Lawrence Trowbridge	National Trust
DD	101-1000	Cambridgeshire	29	Peter Stroh	BSBI
BH	1001-10000	Bedfordshire	34	Corinne Arnold; Sarah Barlow; Kevin Walker; Peter Stroh	RBG Kew; BSBI
KH	1001-10000	Bedfordshire	31	Kevin Walker	BSBI
RC	11-100	Bedfordshire	13	Corinne Arnold; Sarah Barlow; Kevin Walker; Peter Stroh	RBG Kew; BSBI
BA	>10000	Cambridgeshire	33	Kevin Walker	BSBI
BW	>10000	East Gloucestershire	25	Liz Parker	Gloucestershire Wildlife Trust
BD	101-1000	East Gloucestershire	30	Lindsey Pike; Gemma Worswick; Sarah Barlow	RBG Kew
HR	101-1000	East Gloucestershire	34	Corrine Arnold, Sarah Barlow	RBG Kew
HG	101-1000	East Gloucestershire	15	Neil Harris	National Trust
AN[1]	11-100	South Lincolnshire	20	Sarah Evans	Lincolnshire Wildlife trust
SV	1-10	South Lincolnshire	6	Richard Jefferson	Natural England
L	1-10	Mid-West Yorkshire	1	Kevin Walker	BSBI)
MD	1-10	Dorset/Hampshire	9	Linda Smith	Natural England

Table 6 Trial restoration populations of *Anemone pulsatilla L.* sampled for genetic material for inclusion within a population genetic study conducted at RBG Kew, [1]number of generations removed from wild provenance population of original transplants, whilst there was some evidence of recruitment from seed in the HA and Au_au populations, efforts were made to sample from original transplants.

Restored Pop. Code	Provenance (wild) Pop	Restoration Year	Regeneration Cycle[1]	Location	Sample Size	Collector	Collector Affiliation
AR	BA & SH	2013	First	Ashridge Estate (Bedfordshire)	30	Gemma Worswick	RBG Kew
HA	BH	1998	First	Harltslock Reserve (Oxfordshire)	30	Gemma Worswick, Kathy Warden	RBG Kew
AU_au	AU	1999	First	AU	30	Gemma Worswick, Kathy Warden	RBG Kew

Table 7. Ex situ regenerated populations of *Anemone pulsatilla* L. included within RBG Kew population genetic study, [1]'Regenerated' refers to living populations propagated from wild provenance seed and maintained within a nursery settings, [2]number of generations removed from wild provenance population, [3]Wakehurst Place (RBG WP)

Regenerated[1] Pop. Code	Provenance (wild) Pop	Regeneration Year	Regeneration Cycle[2]	Location	Sample Size	Collector	Collector Affiliation
WP(BA)2011	BA	2011	First	WP[3], RBG Kew	30	Gemma Worswick	RBG Kew
WP(BA)2012	BA	2012	First	WP, RBG Kew	30	Gemma Worswick	RBG Kew
WP(BA)2013	BA	2013	First	WP, RBG Kew	30	Gemma Worswick	RBG Kew
WP(BA)2014	BA	2014	First	WP, RBG Kew	30	Gemma Worswick	RBG Kew
WP(BA)2012_F2	BA	2014	Second	WP, RBG Kew	30	Gemma Worswick	RBG Kew
JL(BA)2013	BA	2013	First	Jodrell Lab.,RBG Kew	30	Gemma Worswick, Sarah Barlow	RBG Kew
JL(SH)2013	SH	2013	First	Jodrell Lab.,RBG Kew	30	Gemma Worswick, Sarah Barlow	RBG Kew

3.4 Molecular Genetic Research

3.4.1 DNA Extraction

DNA extraction of leaf samples was carried out using a modified CTAB method (Doyle and Doyle, 1987 – see appendix 1). Extracted total DNA was purified using columns (QIAGEN QIAquick PCR Purification Kit – see appendix 2) and resulted in approximately 2µg of DNA suspended in buffer. Each individual leaf sample was maintained separately in the processes of DNA extraction. Each extraction was labelled with a unique code (retained throughout the process of PCR amplification and microsatellite analysis) that references the population code and a sequential individual sample number.

DNA extractions were carried out at the Jodrell Laboratory (RBG Kew) by Corrine Arnold and Lindsay Pike in 2012 (TH, AU, SH, DD, BH, KH, RC, BA, BW, HR, HG, AN, SV, L, MD) and Gemma Worswick in 2013 (BD, AR, HA, AU_au, WP(BA)2011, WP(BA)2012, WP(BA)2013, WP(BA)2014, WP(BA)2012_F2, JL(BA)2013, JL(SH)2013).

3.4.2 Primer Selection for DNA Fragment Analysis

Corrine Arnold (2012) of RBG Kew developed fourteen primers for A*. pulsatilla* with the aid of next generation sequencing technology. Forward and reverse primers were designed by the QDD pipeline for microsatellite regions (sequence of tandem repeat units typically 1 to 5 base pairs in length) identified form the complete *A. pulsatilla* genome sequenced by Eurofins MWG Operon for 454 FLX+ sequencing using Roche 454 long read technology. Ten nuclear polymorphic primers have subsequently been selected as suitable for inclusion in the statistical analysis of the impact of fragmentation on the population

genetic structure of in situ, restoration intervention, and ex situ regeneration populations of *A. pulsatilla*.

3.4.3 Microsatellite DNA Amplification

PCR for the ten microsatellite regions (loci) yielding scorable polymorphic products was performed with fluorescently labelled primers for all sampled individuals from in situ, restored and ex situ populations. Reaction volumes of 10µl for PCR were made up using Thermo Scientific Fermentas PCR Master Mix for seven of the primers that did not require PCR optimisation. For each 1µl DNA sample the additional 9µl of PCR reaction volume aliquoted out to each reaction tube consisted of 0.2µl of forward primer (fluorescently labelled), 0.2µl of reverse primer, 0.2µl of 0.4% Bovine Serum Albumin (BSA), 5µl of Thermo Scientific Fermentas PCR Master Mix (containing 1mM MgCl2, dNTPs and Taq polymerase), and 3.4µl of nuclease free water. The PCR programme for these primers was set as follows; 94°C for 2 minutes (activation step), 28 cycles at 94°C for 1 minute, 50-58°C for 1 minute, 72°C for 1 minute 30 seconds and a final extension period at 60°C for 20 minutes. For some primers producing weak bands an additional 8 cycle programme was inserted, prior to the final extension period, of 94°C for one minute, 50°C for 1 minute, and 72°C for one minute.

For primers requiring optimisation the additional 9µl of PCR reaction volume aliquoted to reaction tubes with 1µl of DNA consisted of 5µl of QIAGEN Type-It Multiplex PCR Master Mix (3mM MGCl2, dNTPs, HotStarTaq *Plus* DNA Polymerase), 2µl Q solution, 0.2µl of forward primer (fluorescently labelled), 0.2µl of reverse primer, and 1.6µl of nuclease free water. The touchdown PCR programme for these primers required the following programme; activation step of 95°C for five minutes, 18 cycles of 95°C for 30 seconds followed by 58-60°C for 90 seconds followed by 72°C with the annealing

temperature decreasing 0.5°C for every cycle, 20 cycles of 95°C for 30 seconds followed by 49-51°C for 90 seconds, followed by 72°C for 30 seconds, and a final extension period of 60°C for 30 minutes.

PCR amplification was carried out at the Jodrell Laboratory (RBG Kew) by Corrine Arnold and Lindsay Pike in 2012 and 2013 (TH, AU, SH, DD, BH, KH, RC, BA, BW, HR, HG, AN, SV, L, MD) and by Gemma Worswick in 2013 and 2014 (BD, AR, HA, AU_au, WP(BA)2011, WP(BA)2012, WP(BA)2013, WP(BA)2014, WP(BA)2012_F2, JL(BA)2013, JL(SH)2013).

3.4.4 Microsatellite Analysis

PCR products for each sampled individual, and for each of the ten selected microsatellite loci, are separated on 1% agarose gel and prepared for fragment analysis with the use of DNA sequencing technology. The PCR products for each sample are diluted, as appropriate, using nucleotide free water (the required dilution inferred from the strength of bands observed on 1% agarose gel) and suspended in individual 9µl aliquots of Applied Biosystems Grade Hi-Di Formamide with Applied Biosystems GeneScan 500 Rox size standard, in individual wells of a 96 well plate. The suspended fluorescently labelled PCR products are run through an ABI3730 DNA Analyser. GeneMapper software was used to analyse the DNA Analyser output and provide visual confirmation of the correct allele calls for each sampled individual at each of the 10 polymorphic microsatellite loci.

Sequencing machine operated at Jodrell Laboratory (RBG Kew), allele calls used in this analysis made by Corrine Arnold and Lindsay Pike (TH, AU, SH, DD, BH, KH, RC, BA, BW, HR, HG, AN, SV, L, MD) Gemma Worswick (BD, AR, HA, AU_au, WP(BA)2011, WP(BA)2012, WP(BA)2013, WP(BA)2014, WP(BA)2012_F2, JL(BA)2013, JL(SH)2013).

3.5 Population Genetic Analysis

3.5.1 Polysat in R v3.0.2 Console: for the estimation of allelic frequencies and F_{ST} values

The Polysat package (in R v3.0.2 console) includes tools for an exploration of within population genetic diversity and among population genetic variation (Clark 2013). Allelic frequency counts for each individual at each microsatellite locus provide a means to compare within population genetic diversity among in situ populations, and among ex situ regenerated/in situ restored populations and their wild provenance populations. Calculation of pairwise F_{ST} values in Polysat (in R v3.0.2 console) allows for a quantification of among population genetic differentiation (Wright 1978; Hartl & Clark 1997).

Table 8.F_{ST} values as a measure of population genetic differentiation (Hartl & Clark 1997)

Little Differentiation	Moderate Differentiation	Great Differentiation	Very Great Differentiation
0.00 to 0.05	0.05 to 0.15	0.15-0.25	>0.25

Pairwise F_{ST} values are calculated between 0 and 1 and represent the proportion of genetic variation partitioned among, as opposed to within, populations, see table 8. Calculation of F_{ST} values can be applied to confirm the presence of population genetic structure and estimate genetic distance among populations (Balloux & Lugon-Moulin 2002). It is important to note that interpretation of F_{ST} values to estimate extent of population genetic structure varies under different molecular marker scenarios (Balloux & Lugon-Moulin 2002). For example, moderate to high levels of loci polymorphism (as expected for microsatellite markers) will act to reduce F_{ST} expectations so that moderate to great population genetic differentiation will be represented by relatively low F_{ST} values (Wright 1978; Charlesworth 1998; Nagylaki 1998; Hedrick 1999).

3.5.2 Multivariate Analysis of Population Genetic Structure

Principle Component Analysis (PCA), conducted in Polysat package using R v.3.0.2 console, generates a two dimensional graphical representation of a pairwise genetic distance matrix calculated by Lynch Distance (Clark & Jasieniuk 2012). Individual genotypes are assigned a symbol to identify the population of origin and are plotted against the two PCA components (axis) that explain the greatest amount of variance among all sampled genotypes. Principle Coordinate Analysis (Jombart *et al.* 1999), conducted using the PopGenReport package (Adamack & Gruber 2013) in R v3.0.2 console, generates a two dimensional graphical representation of a pairwise Euclidean ('true') genetic distance matrix. PCA and PCoA allows for a visual assessment of patterns, i.e. clustering, in the distribution of genotypes along graphical axes representative of genetic distance, informing hypothesis as to the extent of population genetic structure. It may also be possible to infer, by eye, an association between genetic distance among genotypic clusters and geographic/phenotypic data attached to sampled individuals/populations variables (Jombart *et al*. 1999; Pritchard *et al.* 2000).

Global Spatial Auto-Correlation (Smouse & Peakall 1999), performed in PopGenReport package in R v3.0.2 console, incorporates spatial information (i.e. map coordinates) into multivariate analysis to test for the significance of inferred associations between geographic and genetic distance of populations (such as may be hypothesised from PCA and/or PCoA). Simulated population genetic studies have successfully demonstrated that positive spatial auto-correlation develops quickly among isolated (i.e. restricted gene flow) populations (Smouse & Peakall 1999). Rejecting the null hypothesis of r=0 (i.e. no significant association between genetic and geographic distance) for *A. pulsatilla* in situ

populations could be interpreted as supporting a scenario of genetic drift driving the emergence of population genetic structure across the species' fragmented UK range (Smouse & Peakall 1999).

A key limitation of PCA and PCoA, and other multivariate distance based methods for visualising population genetic structure, is the dependence of observed genetic clustering on the graphical representation and pairwise distance matrix chosen (Pritchard *et al.* 2000). Distance based methods of clustering also lack a measure of confidence (i.e. statistical likelihood) for the observed population genetic clustering (Pritchard *et al.* 2000). It is also not possible to relate the unique sample code for individual genotypes to a PCA/PCoA plot, i.e. to identify introduced and native genotypes of AN.

3.5.3 STRUCTURE, STRUCTURE HARVESTER, AND CLUMPP: a model based approach to population genetic cluster analysis

To achieve fine scale inference of population genetic structure among in situ, restoration trial, and ex situ regenerated populations of *A. pulsatilla* modelling software can be applied to raw genetic marker data to perform genetic cluster analysis (Pritchard *et al.* 2000). This enables us to identify population (and potentially sub population) genomic clusters that are defined by a characteristic set of allelic frequencies. A key advantage of model based approaches to analysis of population genetic structure is the opportunity to vary parameter settings to maximise the potential for estimation of population genetic structure that is a good biological fit to the data. Critically, model based clustering methods allow for an evaluation of the statistical likelihood of different clustering relationships, allowing for a measure of confidence in conclusions drawn regarding the association between genetic distance and geographic/phenotypic sampling data (Pritchard *et al.* 2000).

STRUCTURE v2.3.4 (Pritchard *et al*. 2010) is a widely applied model based clustering approach to the statistical analysis of population genetic structure. STRUCTURE allows raw molecular marker data to be run under a number of: biological assumptions (such as shared (admixed) or distinct (no admix) ancestry of populations); modelling parameters (such as number of burnin iterations and MCMC sampling runs); and, for a number of different K (cluster) values. Under the admixed ancestry model sampled genotypes are assigned cluster membership proportions (Q) that reflect the partitioning of ancestry among genetic clusters. Under the no admix ancestry model sampled genotypes are assigned membership probabilities (Q) for each distinct genetic cluster (Pritchard *et al.* 2010). Cluster membership proportions/probabilities (Q) for sampled genotypes can be graphically represented as vertical bars (collated into a Q bar plot). The vertical bars are composed of proportional length K (cluster) segments with each cluster assigned a unique colour. When including population data within the data set STRUCTURE will also output cluster membership proportions/probabilities for populations defined by the user.

Running a STRUCTURE project allows sampled genotypes to be assigned membership proportions (or probabilities) for multiple runs of numerous assumed K values. Statistical analysis of the likelihood of population genetic structure simulated under different K values is achieved by uploading STRUCTURE project output to STRUCTURE Harvester software (Earl & vonHoldt 2012). Selection of the most appropriate K value for the population genetic data from the likelihood estimations generated by STRUCTURE Harvester requires a degree of subjectivity. This is particularly true for data that lacks discrete populations but which demonstrates subtle population structure with a degree of admixed ancestry among

populations, as is a common feature of real populations (Waples *et al.* 2006; Pritchard *et al.* 2010).

STRUCTURE HARVESTER provides a graphical representation of two statistical estimates of the likelihood of different K values; a graph of mean L(K) values - Estimated Ln Prob of Data – generated post hoc by STRUCTURE, and a graph of Delta K values - second order rate of change of L(K)/(SD(L(K)) - calculated using the Evanno *et al.* (2005) method. In general, the Delta K criterion produces more conservative estimates of the most likely K value than L(K). Where populations are discrete (a feature more generally associated with simulated as opposed to real population genetic data) Delta K provides the most appropriate likelihood estimate to identify the real K value and to avoid overestimation of population structure (Pritchard *et al.* 2010). However, a common feature of real population genetic data is the incorporation of a degree of admixture (as a correlate of geographic distance) with the result that K is not a definitive quantity (Pritchard *et al.* 2010). In this case, it is appropriate to consider a number of K values as potentially providing a valid description of population genetic structure (Waples *et al.* 2006; Pritchard *et al.* 2010). For real population data it is often more appropriate to consider the K values at the peak of the L(K) graph, which are generally higher than the peak K value on the Delta K graph, as the most likely descriptors of population genetic structure. This approach can be justified when clusters (K) make biological sense and membership proportions/probabilities (Q) estimated for individual genotypes are in general strongly biased towards a single genetic ancestry cluster (Waples *et al.* 2006; Pritchard *et al.* 2010).

When considering L(K) values as an estimate of population genetic structure it its common that a number of K values will show similar likelihood values at the peak of the L(K)

curve (Pritchard *et al.* 2010). To avoid overestimation of K from L(K), it is advised that when a number of K values show similar high likelihood and low variance estimates the smallest of these K values should be selected to explain the majority of population genetic structure (Pritchard *et al.* 2010).

Following selection of an appropriate K value, STRUCTURE Harvester output can be imported into the software CLUMPP which aligns individual and population cluster membership proportions/probabilities (Q) estimated under all STRUCTURE iterations for the selected K value. CLUMPP v1.2.2 (Jakobsson & Rosenberg 2009) output can be imported into EXCEL or a graphical programme such as STRUCTURE or DISTRUCT to visualise the resultant Q bar plots of averaged K membership proportions/probabilities for individuals and populations. Each individual Q bar of cluster membership proportions retains the sequential sample number applied to the original DNA extraction, thus enabling individuals within a population to be traced back to the original leaf sample.

3.5.4 STRUCTURE Project Modelling Parameters

For an analysis of *A. pulsatilla* in situ population genetic structure it was determined that assumption of an ancestral relationship among populations (admixed) and assumption that variation in allelic frequencies among populations can be accounted for by genetic drift and founder effect as an artefact of fragmentation (correlated allele frequencies) were the best biological fit for the data. The cluster membership proportions assigned therefore represent the extent to which an individual's genotype is estimated to be partitioned among defined genetic ancestry clusters.

To confirm the presence of population genetic structure across the in situ UK population of *A. pulsatilla* STRUCTURE was run initially for genotypes composed of 10 microsatellite loci from 16 sampled in situ populations (331 individual total) without sampling location data (i.e. population of origin). Model parameters of 10,000 burnin iterations and 10,000 Monte Carlo (MCMC) sampling reps were chosen with 20 runs for each K value. Post hoc analysis of STRUCTURE output by STRUCTURE Harvester identified a plateau of L(K) values from K=5 to K=7 whilst Delta K identified 2 as the highest likelihood value (figure 5). CLUMPP averaged membership assignments (Q) for individual and population bar plots at K=5 conformed to expectations for real population genetic structure (i.e. individual membership proportions strongly biased towards a particular genetic ancestry cluster).

Subsequently, a STRUCTURE project was set up for 16 in situ populations with sampling location data included. Inclusion of sampling location data assists clustering of real populations, where admixture and correlated allele frequencies is a general feature. The addition of sampling location data allows for the detection of a weaker signal of population genetic structure without compromising the integrity of the model (Pritchard *et al.* 2010). Model parameters chosen for the project were 100,000 burnin iterations and 100,000 MCMC sampling reps with 20 runs for each K value between 1 and 8. A second STRUCTURE project was set up to include three restoration trial and their wild provenance populations to allow for an analysis of how populations subject to restoration intervention fit into the *A. pulsatilla* natural population genetic structure. A third STRUCTURE project was set up for ex situ regenerated populations and their wild provenance populations to allow for an understanding of the influence of ex situ regeneration practices on the structuring of natural

genetic variation. Model parameters chosen for these projects were 100,000 burnin iterations and 30, 000 MCMC sampling reps with 20 runs for a range of appropriate K values. Each STRUCTURE project was uploaded to STRUCTURE harvester to allow for a visualisation of the likelihood scores (L(K) and Delta K) for each K value and provides output files compatible with CLUMPP.

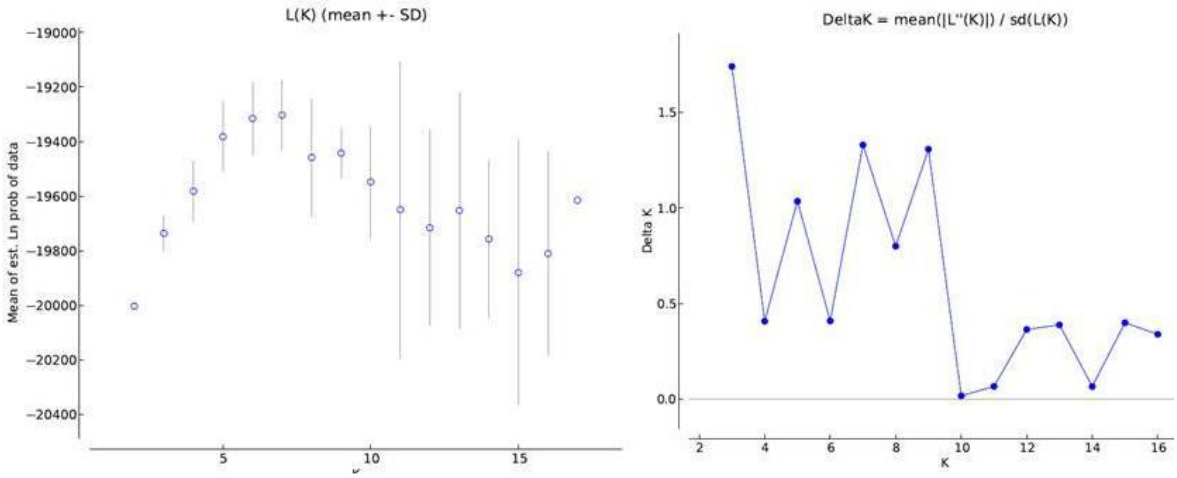


Figure 5. Statistical K likelihood values for 16 in situ populations of *A. pulsatilla,* STRUCTURE assumptions were for admixed ancestry with correlated allele frequencies, sampling locations were not given to test for real population structure, model parameters used were 10,000 burin iterations with 10,000 MCMC sampling reps and 20 runs of each K value between 1 and 16.

All analysis reported in this thesis carried out by Gemma Worswick.

4 Results

4.1 Population Genetic Structure of *Anemone pulsatilla* L. In situ UK Population

4.1.1 Multivariate Analysis (PCA/PCoA and Global Spatial Auto-Correlation)

Multivariate analysis of population genetic data allows for an exploration of patterns of quantified differentiation (i.e. genetic distance) among individuals and populations, which may be explained by associated ecological and/or biological variables. Principle Component Analysis (PCA) and Principle Coordinate Analysis (PCoA) provide graphical representations of pairwise genetic distance matrices calculated for *A. pulsatilla* genotypes representative of the species' extant in situ UK distribution. PCA distributes sampled individuals along the two axes (components) which explain the greatest amount of variation among genotypes (figure 6). The distribution of individuals along the axes of the PCoA graph represents Euclidean (i.e. 'true') genetic distance among genotypes (figure 7). Global Spatial Auto-Correlation provides a statistical test of the dependent association among two distance variables, such as genetic and geographic distance, among *A. pulsatilla* genotypes sampled from natural populations (figure 8).

Principle Component Analysis (PCA)

The distribution of genotypes along the 1st and 2nd PCA axes is indicative of the emergence of real population genetic structure among the 331 *A. pulsatilla* individuals sampled across 16 in situ populations (figure 6). A broad geographic trend can be observed in the continuous distribution of genotypes along the 1st PCA axis. For example, genotypes of western range populations (HR, BD, HG and BW) can be seen to cluster distinctly towards the right of the 1st PCA axis whilst genotypes of eastern range populations (BA, DD, BH, KH,

TH and RC) are distributed continuously from the centre to the left extreme of the 1st PCA axis. Further structuring of genetic variation can be observed among western range populations as the HR, BD genotypes form a distinct cluster at the extreme right of the 1st PCA axes whist BW, HG genotypes are distributed closer to the centre of the 1st PCA axes (although still with a right bias). Of the eastern region populations distributed from the centre to the left of the 1st PCA coordinate, BA genotypes demonstrates the most extreme left bias. A geographic trend in the distribution of southern and northern range populations is less apparent. Structuring of genetic variation among southern range populations can be inferred apparent as AU genotypes demonstrate a bias in distribution towards the extreme left of the 1st PCA axis, overlapping with BA, whilst SH and MD (a casual introduction on unknown genetic provenance) genotypes are demonstrate a bias in distribution towards the centre of the 1st PCA, overlapping to a degree with the western range BW, HG genotype cluster. Genotypes of the northern range populations AN & SV also demonstrate a bias in distribution towards the centre of the 1st PCA axis.

There is in general little separation of populations along the 2nd PCA axis with the exception of a discrete partitioning of a sub-set of genotypes assigned to the northern range population AN (the subject of a casual augmentation) that are discreetly clustered at the lowest extreme of the axis. A less distinct clustering of genotypes of the southern range AU population can be observed along the upper extreme of the 2nd PCA axis.

Principle Coordinate Analysis (PCoA)

To generate the PCoA graph the genotypes of 12 in situ populations were included in the analyses, the very small population L (one individual) and populations with outlying genotypes (SV, MD and AN) were excluded to allow for scale. Reflecting trends observed

from the PCA graph the distribution of *A. pulsatilla* genotypes along the 1st and 2nd PCoA axes broadly reflect the geographic groupings of natural populations. For example, along the 1st and 2nd PCoA axis the genotypes of eastern region populations BH, KH, RC and TH, representing the centre of extant *A. pulsatilla* UK population density, are biased towards a central distribution. Genotypes of western region populations HR, BD, HG and BW are distinctly distanced from eastern region populations along the 1st PCoA axis. Reflecting trends observed from PCA analysis the western range populations differentiate into two distinct genotype clusters along the 1st PCoA axis, the HR, BD cluster occupying the extreme left of the 1st PCoA axis and the HG, BW cluster occupying a centre left distribution. Genotypes of the eastern region BA population are distanced from other populations along the 1st and 2nd PCoA axes. Along the 2nd PCoA axis BA is clustered away from other eastern region populations, more discretely than observed in the PCA graph. Along the 1st PCoA axis BA genotypes occupy an extreme right distribution, the opposite extreme of the western region HR, BD cluster.

Global Spatial Auto-Correlation

A significant positive spatial auto-correlation can be observed between pairwise genetic and geographic distances for *A. pulsatilla* genotypes representing natural populations (figure 8). This dependent association of genetic and geographic distance variables provides statistical support to the broad geographic trend in population genetic differentiation inferred from the distribution of genotypes in PCA and PCoA graphs (figures 6 & 7). MD and AN are excluded from this analysis due to unverified provenances of introduced genotypes, natural populations L and SV are excluded due to small sample size.

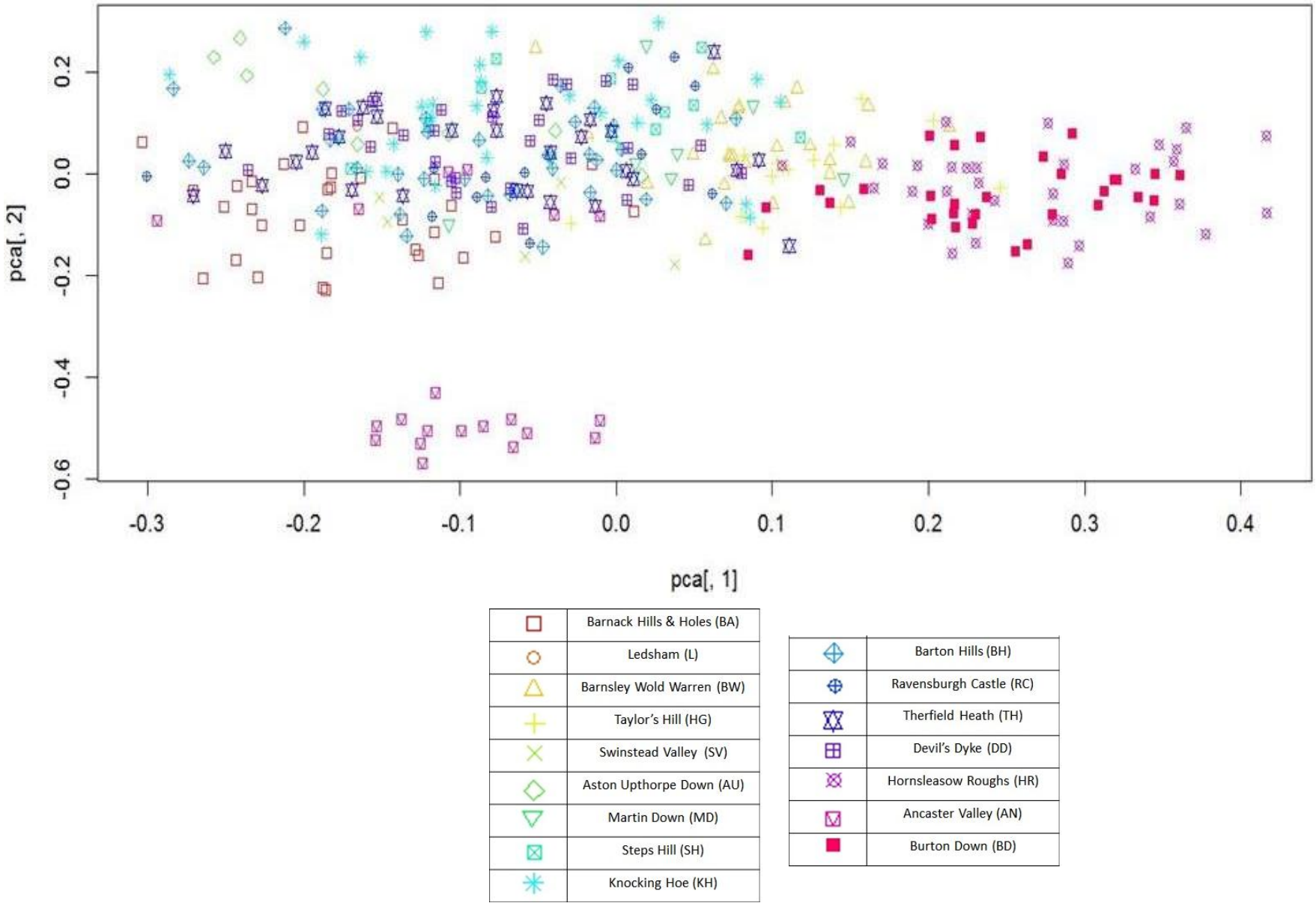


Figure 6. Principle Component Analysis (PCA) performed by Polysat in R from for 10 loci microsatellite genotypes sampled across 16 in situ UK populations of *Anemone pulsatilla L.*, individuals are dispersed along the two axes which explain the greatest amount of variance among microsatellite genotypes, variance among genotypes is calculated from a pairwise genetic dissimilarity matrix, Lynch Distance = 1- (number of alleles in common)/(average number of alleles/ genotype).

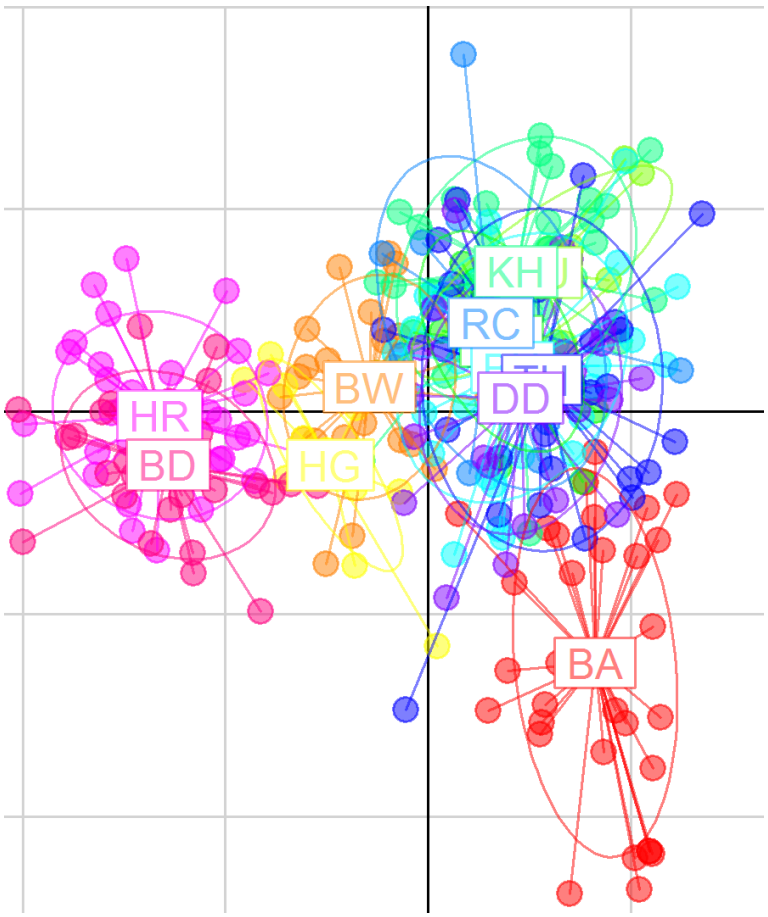


Figure 7. Principle Coordinate Analysis (PCoA) performed by PopGenReport in R from for 10 loci microsatellite genotypes sampled across 12 in situ UK populations of *Anemone pulsatilla L.*, outlying populations L, SV, MD and AN are removed from the analysis to allow for scale, individuals are dispersed along the two PCoA axes to reflect Euclidean (true) distance among points (genotypes).

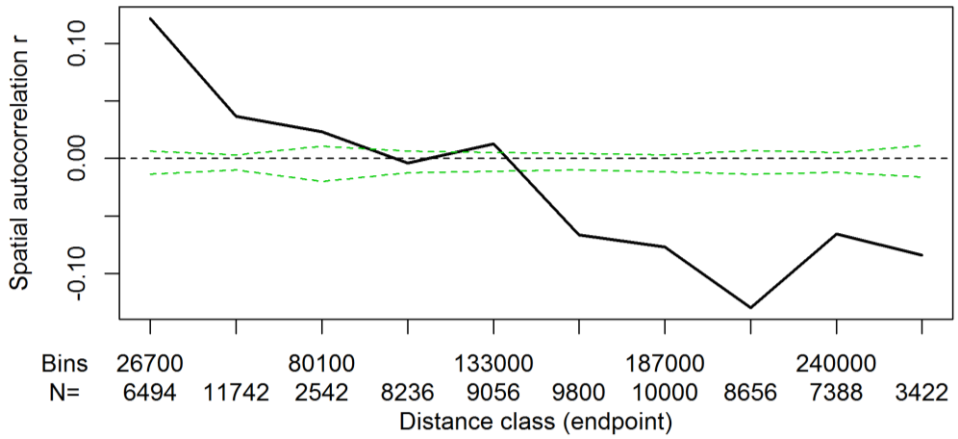


Figure 8. Global Spatial Auto-correlation of 12 in situ populations of *A. pulsatilla*, outlying populations L, SV, MD and AN are excluded from the analysis, r = the Auto-correlation coefficient (calculated for each pairwise genetic distance pair at each geographic distance class), r = 0 represents the null hypothesis of no dependent association among genetic and geographic distance, the null hypotheses can be rejected for this graph.

4.1.2 STRUCTURE: A Modelling Approach to Cluster Analysis

Post hoc analysis of the STRUCTURE project modelling *A. pulsatilla in situ* population genetic structure at a range of assumed cluster (K) values demonstrates a steady increase in mean likelihood probability L(K) from K=1 through all K values to a plateau of L(K) between K=5 and K=8 (figure 9). The Evanno method (Delta K) for estimating the most probable K value, as calculated by STRUCTURE Harvester, demonstrates peak likelihood at K=3 (figure 7). The most conservative cluster (K) value that can be considered to describe the partitioning of genetic variation among UK populations of *A. pulsatilla* is therefore K=3.

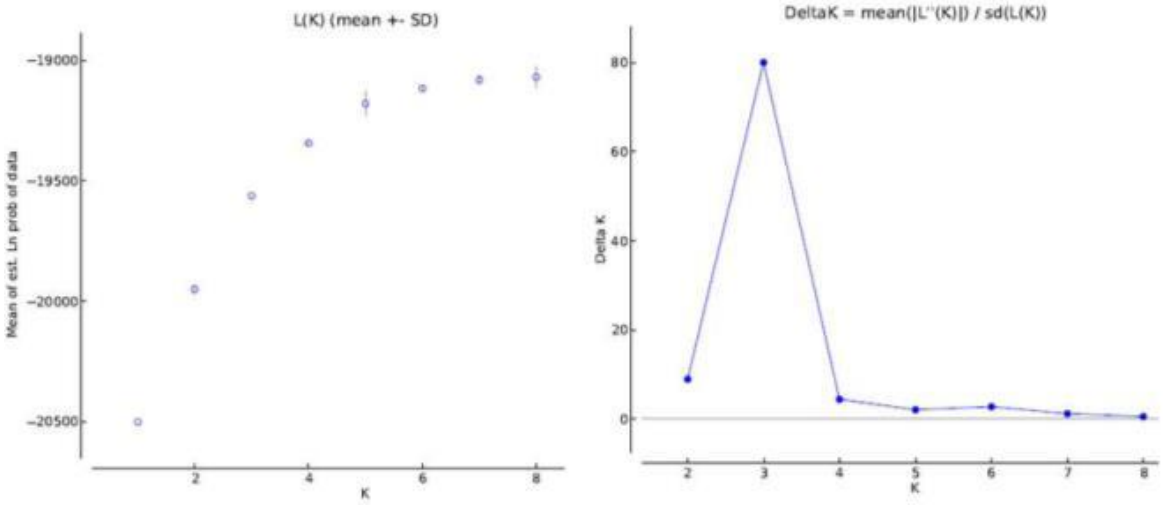


Figure 9. STRUCTURE Harvester generated statistical cluster (K) likelihood values for 16 in situ populations of *A. pulsatilla*, STRUCTURE project run for K=1 to K=8 (20 iterations each), parameter set: admixed (ancestry), correlated (allele frequencies), 100,000 iterations for burin period and 100,000 iterations for Monte Carlo reps), sampling locations included in data set to assist detection of subtle genetic structure.

Pritchard *et al.* (2010) recommends that for STRUCTURE modelling of population genetic clustering among real populations, i.e. where a degree of admixed ancestry is likely to occur, that L(K) (as opposed to Delta K) is the appropriate guide to selecting the most likely K value. When inferring an appropriate cluster (K) value from a graph of mean L(K) likelihood probabilities it is sensible to choose the K value at the base of a plateau of L(K)

values occurring at peak likelihood (Pritchard *et al.* 2010). In the case of inferring population genetic structure across the UK in situ distribution of *A. pulsatilla*, K=5 represents the lowest value at the base of the plateau of L(K) values and therefore appears to be a sensible choice for describing the partitioning of genetic variation among UK populations. A caveat to justifying a cluster (K) value greater than is indicated by the peak Delta K is that population genetic clusters must make biological sense, i.e. the partitioning of genetic variation among populations and population genetic clusters would be expected to be associated with geographic, ecological, and/ or phenotypic variables. CLUMPP averaged STRUCTURE output in the form of Q bar plots provide graphical representation of genetic cluster membership proportions for sampled genotypes, or for sampled populations (i.e. cluster membership proportions averaged across a set of individuals defined by the user). From population Q bar plots generated for sequential K values, from K=2 to K=5, it is possible to explore the development of population genetic structure across the fragmented range of *A. pulsatilla* and assess the biological fit of clustering relationships that emerge (figures 10 & 11).

At K=2 population genetic structure can be seen to develop as the partitioning of western range edge populations HR and BD (blue cluster) away from the main population genetic cluster (yellow cluster) which is defined by eastern range population (i.e. the extant centre of population density in the UK). Populations that display admixed ancestry between the eastern and western range genetic cluster at K=2 include the two northern range populations AN (a casual augmentation) and SV, the southern range population MD (a casual introduction), and the western range populations HG and BW. At K=3 population genetic structure is seen to develop as the partitioning of northern range population AN (pink cluster). At K=4 population genetic structure is seen to develop as the differentiation

of BA (green cluster). Other eastern range populations (DD, BH, KH, TH and RC), southern range population AU, and northern range populations AN, SV and L also assign a significant proportion of genetic ancestry to the fourth emergent cluster. At K=5 population genetic structure is seen to develop as the partitioning of western range populations HG and BW and the southern range populations MD and SH to a fifth emergent genetic cluster (red cluster). At K=5, eastern range populations DD, BH, KH, TH and RC are assigned a much reduced membership proportion for the fourth emergent genetic cluster (green) and are assigned predominate membership of the main genetic cluster (yellow). The northern region population SV is partitioned predominately to the fourth emergent genetic cluster (green) with BA.

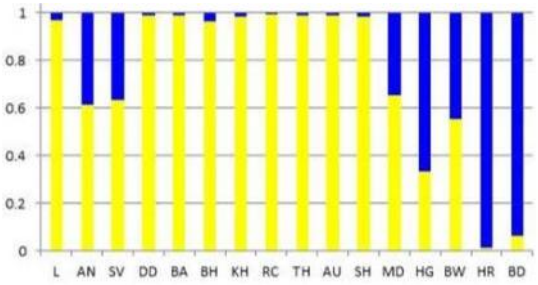


Figure 10a. Population Genetic Clusters, K=2

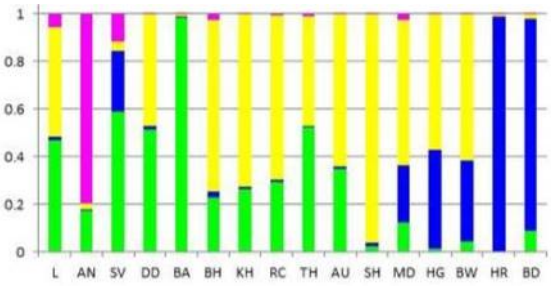


Figure 10c. Population Genetic Clusters, K=4

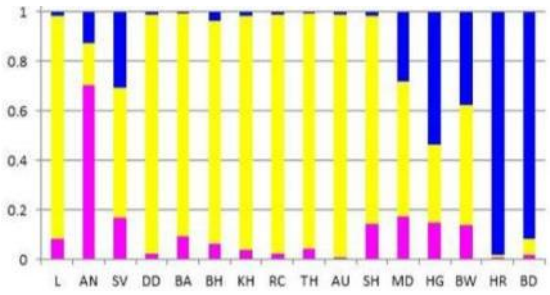


Figure 10b. Population Genetic Clusters, K=3

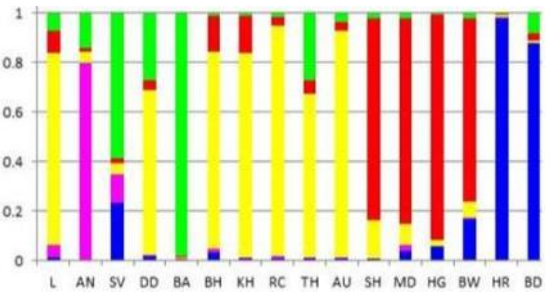


Figure 10d. Population Genetic Clusters, K=5

Figure 10. CLUMPP averaged STUCTURE output for UK populations of *A. pulsatilla* at K=2 (a), 3 (b), 4 (c) and 5 (d), STRUCTURE project run for K=1 to K=8 (20 iterations each), parameter set: admixed (ancestry), correlated (allele frequencies), 100,000 burin period iterations and 100,000 Monte Carlo sampling reps, division of population bars into coloured segments represents cluster membership proportions (Q), i.e. division of Q represents the extent to which genetic ancestry of a population can be assigned to different genetic clusters, populations are arranged to reflect the geographic trends in the distribution of *A. pulsatilla* UK populations.

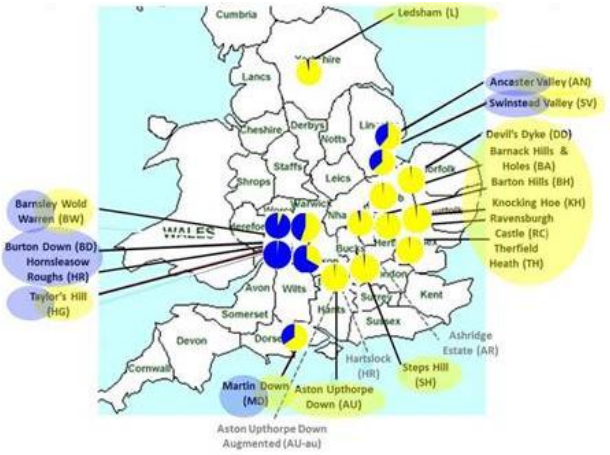


Figure 11a. Population Genetic Clusters, K=2

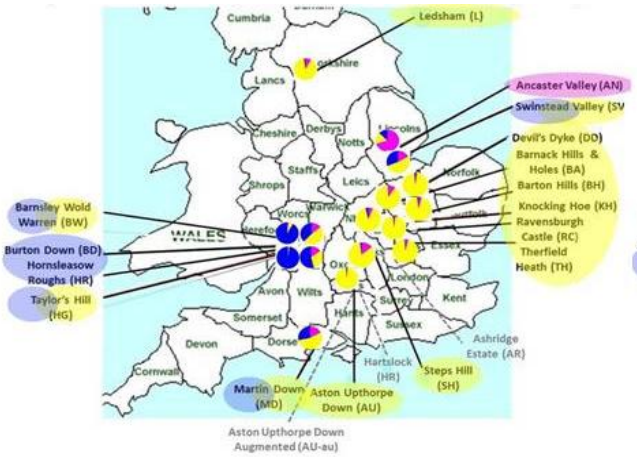


Figure 11b. Population Genetic Clusters, K=3

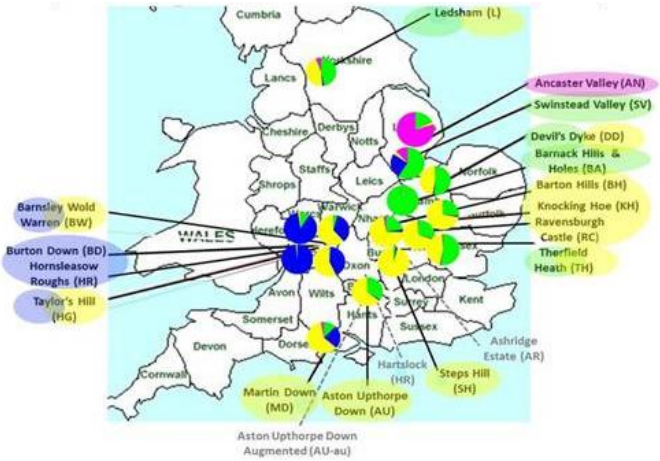


Figure 11c. Population Genetic Clusters, K=4

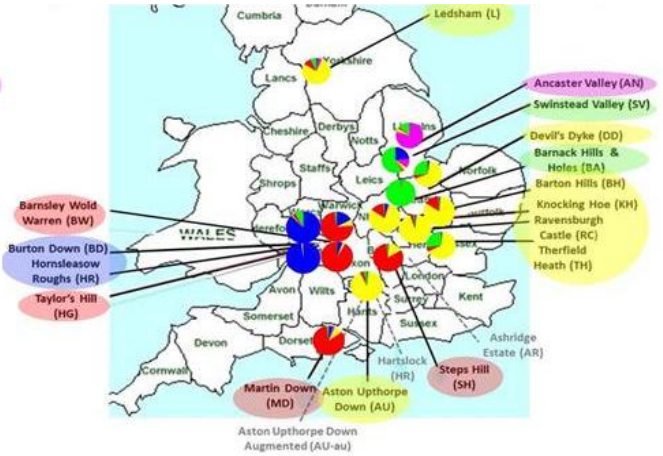


Figure 11d. Population Genetic Clusters, K=5

Figure 11. Population genetic clustering across the UK distribution of *A. pulsatilla* for assumed K values of 2 (a), 3 (b), 4 (c) and 5 (d), pie charts represent CLUMPP averaged STRUCTURE estimated membership proportions for populations, map adapted from Walker (2011)

The development of population genetic structure across the UK range of *A. pulsatilla* from K=2 to K=5 can be interpreted to reflect geographical patterns of historical range fragmentation and the impact of causal restoration intervention. CLUMPP estimated structure output at K=5 can also be seen to reflect spatial trends in the distribution of genotypes along the genetic distance axes of PCA and PCoA (figures 6 &7). Selection of K=5 as the lowest cluster value to explain the greatest amount of *A. pulsatilla* UK population genetic structure, as estimate by the L(K) graph of likelihood probability, therefore fits with biological expectations.

CLUMPP averaged STRUCTURE output for individual cluster membership assignments (Q bar plots) at K=5 provides an insight into the impact of casual restoration intervention on *A. pulsatilla* UK population genetic structure (figure 12). Cluster membership proportions (Q) are generally consistent among individuals within natural in situ populations. However, sub-structuring of genetic variation can be observed among genotypes assigned to the casual restoration intervention populations AN and MD. At K=5, six AN genotypes are partitioned out of the main genetic cluster for this population, reflecting the separation of AN genotypes observed along the 2nd PCA axis (figure 6).One MD genotype is partitioned out of the main genetic cluster for this population at K=5.

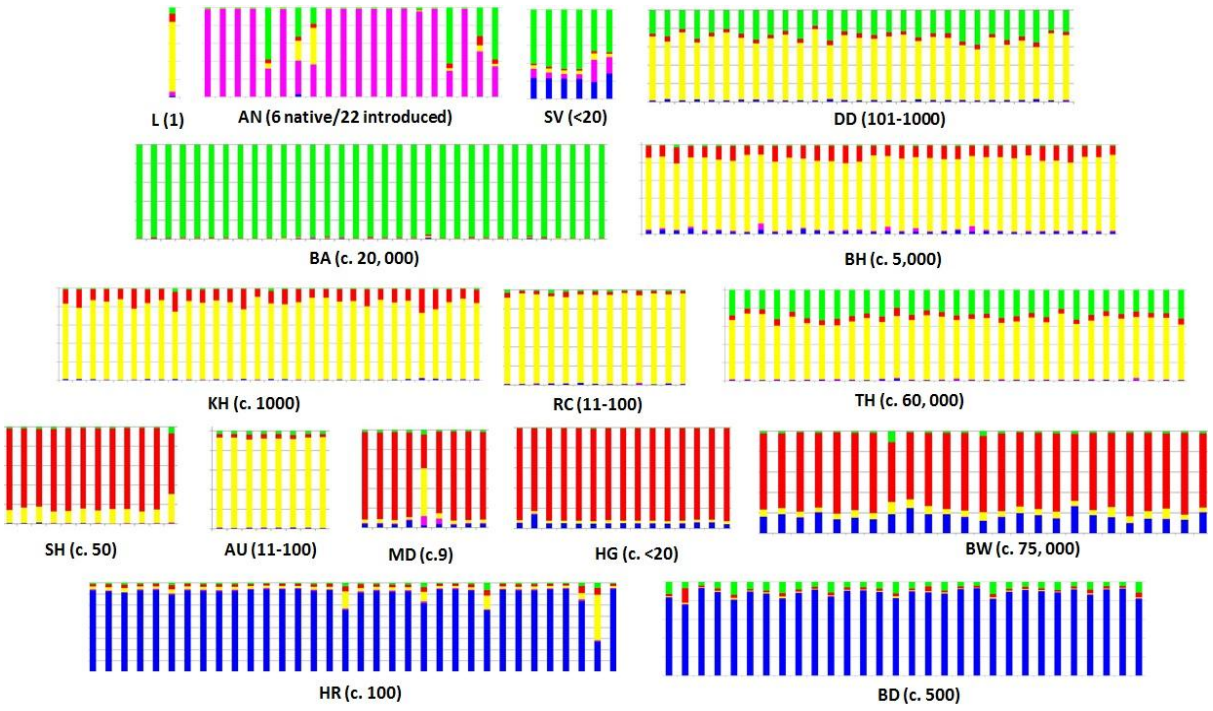


Figure 12. CLUMPP averaged STUCTURE output for individuals sampled across the UK distribution of *A. pulsatilla* at K=5, STRUCTURE project run for K=1 to K=8 (20 iterations each), parameter set: admixed (ancestry), correlated (allele frequencies), 100 000 burin period iterations and 100 000 Monte Carlo sampling reps.

4.1.3 Population Genetic Differentiation (F_{ST})

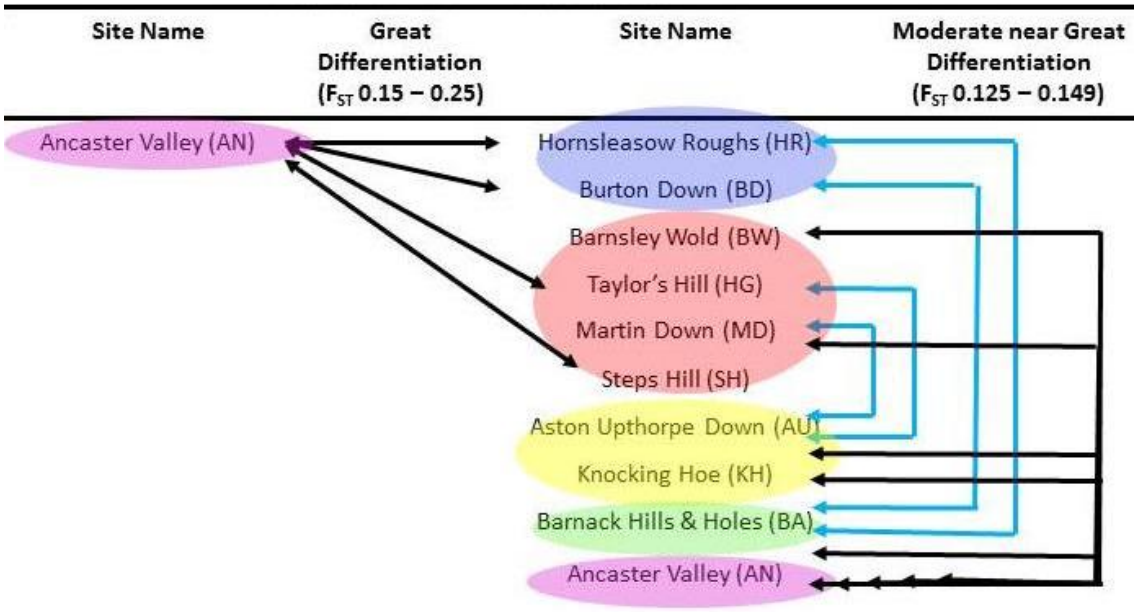
Polysat package in R v3.0.2 console allows for the generation of pairwise F_{ST} values to provide a quantification of population genetic differentiation (Clark 2013). F_{ST} can be applied to identify genetically diverged (i.e. significantly differentiated) populations and provide confirmation of population genetic structure inferred from graphical representations of the partitioning of genetic variation among populations (i.e. PCA, PCoA and STRUCTURE clustering analysis).

Across the in situ distribution of *A. pulsatilla* low (F_{ST} = 0 to 0.05) to moderate (F_{ST} = 0.05 to 0.15) genetic differentiation emerged as the norm for the majority of pairwise comparisons (appendix 3). Great genetic differentiation (F_{ST} = 0.15 to 0.25), and near great genetic differentiation (F_{ST} = 0.125 to 0.149) was observed among a small number of populations (table 9). Given the high levels of loci polymorphism (diversity of alleles at microsatellite loci) recorded for the majority of populations included within this study (appendix 4) it is probable that the pairwise F_{ST} values calculated for *A. pulsatilla* populations underestimate the extent of population genetic differentiation (Balloux & Lugon 2002).

The northern range population AN demonstrates the greatest genetic divergence of all UK populations; great to near great genetic differentiation can be observed among AN and populations representative of all 5 genetic clusters identified by STRUCTURE. The southern range population AU can be observed to record near great genetic differentiation from the western range population HG and the southern range, casual introduction, MD. Near great genetic differentiation can also be observed among western range populations HR, BD and the eastern range population BA. Pairwise F_{ST} calculations therefore reflect

trends inferred from multivariate and STRUCTURE analysis of *A. pulsatilla* population genetic structure for the partitioning of genetic variation among populations to reflect geographic patterns of historical range fragmentation and the impact of casual restoration intervention (figures 6, 7 & 11).

Table 9. Great to near great population genetic differentiation among in situ UK populations of *A. pulsatilla,* F_{ST} 0.15 – 0.25 indicates that a great amount of genetic variation is distributed among populations, F_{ST} 0.05 – 0.15 indicates a moderate amount of genetic variation, F_{ST} values calculated by Polysat in R. Colours represent the population genetic cluster (K) assignment at an assumed K= under SRUCTURE analysis, populations are assigned to a cluster (K) based on the predominate population membership assignments (Q).



4.3 Within Population Genetic Diversity of UK Populations of *Anemone pulsatilla* L.

Allelic diversity counts (loci polymorphism), i.e. the diversity of alleles recorded at each microsatellite loci across all sampled genotypes within a population (calculated by Polysat in R v3.0.2 console), provide a means to quantify within population genetic diversity (figure 13). Unique microsatellite alleles (i.e. alleles that occur in just one population) are recorded from raw microsatellite data for each individual at each microsatellite loci (appendix 4).

Rarefaction analysis can be applied to provide statistical analysis of the influence of sample size on representation of population genetic diversity (i.e. by removing individuals one by one from sampled populations and analysing the impact genetic diversity). However, of the statistical packages available for rarefaction analysis (i.e. vegan in R, FSTAT, and Heirfstat) there does not appear to be an open source package available which can interpret polyploid data files.

4.3.1 Allelic Diversity

For in situ populations sampled across the UK distribution of *A. pulsatilla* mean loci polymorphism (i.e. population allelic diversity count averaged across 10 loci) can be observed to vary from a minimum of 2.2 alleles/microsatellite loci (Ledsham) to a maximum mean loci polymorphism of 7.9 alleles/microsatellite loci (Therfield Heath). Across all in situ populations sampled the mean loci polymorphism is observed to be recorded as 5.1 alleles/microsatellite loci.

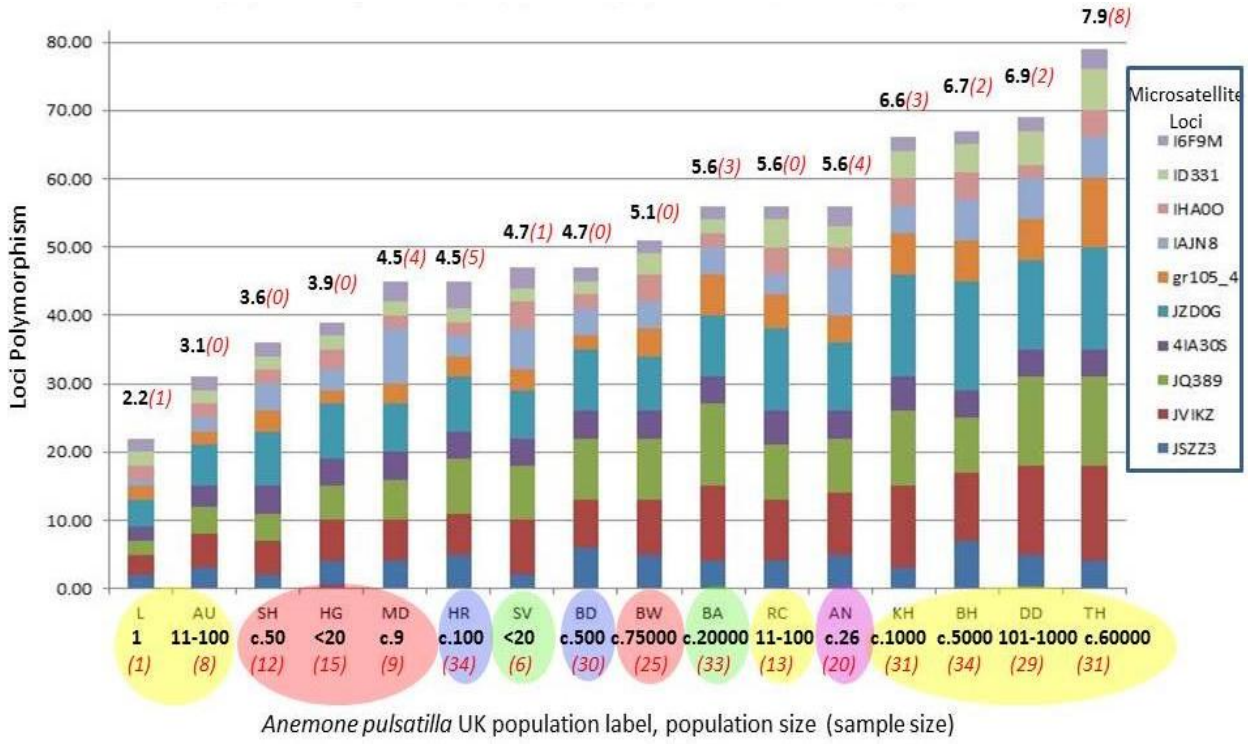


Figure 13. Allelic diversity counts calculated by Polysat in R for 10 microsatellite loci across 16 of the 19 UK populations of *Anemone pulsatilla L.*, mean loci polymorphism (allelic diversity averaged across all 10 loci) recorded for each population in numeric above allele count bars with number of unique alleles attributed to each population recorded in brackets, along the x axis populations are coded with the colour of the STRUCTURE estimated cluster membership at K=5, population size is recorded beneath the population label with sample size for population genetic study recorded in brackets.

A broad association between population size and within population genetic diversity (i.e. loci polymorphism) can be observed for in situ UK populations of *A. pulsatilla*. Across the five largest UK population sites (BH, KH, BH, TH and BW), accounting for >99% of *A. pulsatilla* UK population, the mean loci polymorphism count is recorded as 6.4 alleles/microsatellite loci, greater than the UK population average. For the nine populations sampled that number <100 individuals the mean loci polymorphism count is recorded as 4.2 alleles/microsatellite loci, fewer than the UK population average.

Within population genetic diversity can also be observed to vary among geographical regions of *A. pulsatilla* UK range. For population sites located in the eastern region of the species' range (BA, DD, BH, KH, RC, and TH), forming the extant centre of population density in the UK, mean loci polymorphism is recorded as 6.6 alleles/microsatellite loci, greater than

the UK population average. For population sites located in the western region of the species' range (HR, BD, HG and BW), an historical centre of population density in the UK that has subsequently experienced a high level of fragmentation, mean loci polymorphism is recorded as 4.6 alleles/microsatellite loci, fewer the UK population average. For population sites located in the fragmented southern region of the species' extant range (SH, AU and casual introduction MD), mean loci polymorphism is recorded as 3.7 alleles/microsatellite loci. For population sites located in the fragmented northern region of the species' extant range (L, SV and casually augmented AN) mean loci polymorphism is recorded as 4.2 alleles/microsatellite loci. Both southern and northern regions of the species' extant range therefore recorded mean loci polymorphism values fewer than the UK population average, with southern populations displaying the lowest allelic diversity counts across all geographic regions of the species' extant range.

Closely, although not definitively, associated with geographical regions of *A. pulsatilla* UK range, within population genetic diversity can also be observed to vary among STRUCTURE estimated population genetic clusters at K=5. The main genetic cluster (yellow), predominately defined by eastern range populations (DD, BH, KH, RC and TH), records a mean loci polymorphism count of 5.6 alleles/microsatellite loci, greater than the population average. At 5.2 alleles/microsatellite loci, the mean loci polymorphism count recorded for the BA, SV genetic cluster (green), located in the north eastern region of the species' UK range, approximates the UK population average. Of the differentiated western region genetic clusters, a mean loci polymorphism count of 4.6 alleles/microsatellite is recorded for the HR, BD (blue) cluster whilst 4.3 alleles/microsatellite loci is recorded for the BW, HG, SH and MD cluster (red). Mean loci polymorphism counts for western region genetic clusters

are therefore lower than the UK population average. The population genetic cluster defined by the restoration intervention AN (pink) records a mean loci polymorphism count greater than the UK population average at 5.6 alleles/microsatellite loci.

4.3.2 Private (Unique) Alleles

Across the UK distribution of *A. pulsatilla* the number of unique microsatellite alleles recorded/population ranges from 0 to 8. The mean number of unique population alleles recorded across the species' UK range is 2.1 alleles/population (with a mode of 0). A general association between population size and unique population genetic diversity can be observed. The mean number of unique population alleles recorded for the five largest UK populations is 3.2 alleles/population (ranging from 0 to 8 with a mode of 3), greater than the UK population average. The mean number of unique population alleles recorded for the nine populations sampled which number <100 individuals is 1.7 alleles/population (ranging from 0 to 5 with a mode of 0), less than the UK population average.

Variation in level of unique population diversity can also be observed among regions within the species' extant UK range. For population sites located in the eastern region of the species' range the mean number of unique population alleles is recorded as 3 alleles/population (ranging from 0 to 8 with modes of 2 and 3), greater than the UK population average. For population sites located in the western region of the species' range the mean number of unique population alleles is recorded as 1.3 alleles/population ranging from 0 to 5 with a mode of 0). For population sites located in the southern region of the species' range the mean number of unique population alleles is recorded as 1.3 alleles/population (ranging from 0 to 4 alleles with a mode of 0). For population sites located in the northern region of the species' range mean number of unique population

alleles is recorded as 2/alleles population (ranging from 1 to 4 with a mode of 1). Northern, western and southern regions of the species' range therefore record unique population diversity counts fewer than the UK population average.

Unique population genetic diversity counts can also been seen to vary among STRUCTURE estimated population genetic clusters, at K=5. For the main UK genetic cluster (yellow) the mean number of unique population alleles is recorded as 2.3 alleles/population (ranging from 0 to 8 with a mode of 2), similar to the UK population average of 2.1. For the BA, SV (green) genetic cluster 3.5 alleles/population is recorded (ranging from 1 to 3), far exceeding the UK population average. For the differentiated western region genetic clusters mean unique population allele counts are recorded as 2.5 alleles/ population (with a range of 0 to 5) for the HR, BD cluster (blue), greater than the UK population average, and 1 allele/ population (with a range of 0 to 4 and a mode of 0) for the BW, HG, SH and MD cluster (red). As a caveat it should be noted that for the red cluster the only population to record unique population alleles is the casually introduced population MD. The population genetic cluster defined by the restoration intervention AN (pink) records a unique population allele count of 4 alleles/population, far exceeding the UK population average

4.4 Population Genetic Structure of Restoration Trial Populations

The populations Hartslock (HA), Aston Upthorpe Down augmented (AU_au), and Ashridge Estate (AR) have been subject to restoration interventions as part of formal restoration trials. HA represents an introduced population located within the historical UK range of *A. pulsatilla,* the population has been restored with the introduction of transplants (i.e. immature plants) that originate from the ex situ regeneration of Barton Hills (BH) wild provenance seed. Transplants introduced to HA represent first generation (F1) ex situ

propagated individuals, i.e. one generation removed from the wild provenance population. AU_au represents augmentation of the in situ UK population Aston Upthorpe Down (AU), where introduced AU_au transplants are established within an enclosure, separate from the native AU population. Introduced transplants originate from the ex situ regeneration of AU wild provenance seed. AU_au transplants represent F1 ex situ propagated individuals, one generation removed from the wild provenance population. The AR introduced population is located within the historical range of *A. pulsatilla,* the population has been restored via in situ regeneration of Steps Hill (SH) and Barnack Hills and Holes (BA) wild provenance seed. The AR population is therefore one generation removed from wild provenance populations.

STRUCTURE Harvester analysis of the STRUCTURE project for restoration trial populations and wild provenance populations demonstrate a steady increase in L(K) likelihood probability from K=1 to a plateau at K=3 (figure 14). Delta K estimation of likelihood probability also peaks at K=3. From population Q bar plots at K=3, it can be observed that restoration intervention populations AU_au and HA are assigned distinct membership of the genetic cluster occupied by the respective wild provenance population (figure 14). The restoration intervention population AR, known to be of admixed ancestry, can be observed to be clustered predominately with the provenance population BA. From the individual Q bar plots for restoration trial and wild provenance populations a partitioning of AR genotypes into two distinct genetic clusters can be observed (figure 15). Seven AR genotypes are assigned predominate membership of the ancestry cluster occupied by the wild provenance population SH whilst the remaining 53 sampled genotypes are assigned predominate membership of the BA wild provenance population genetic cluster.

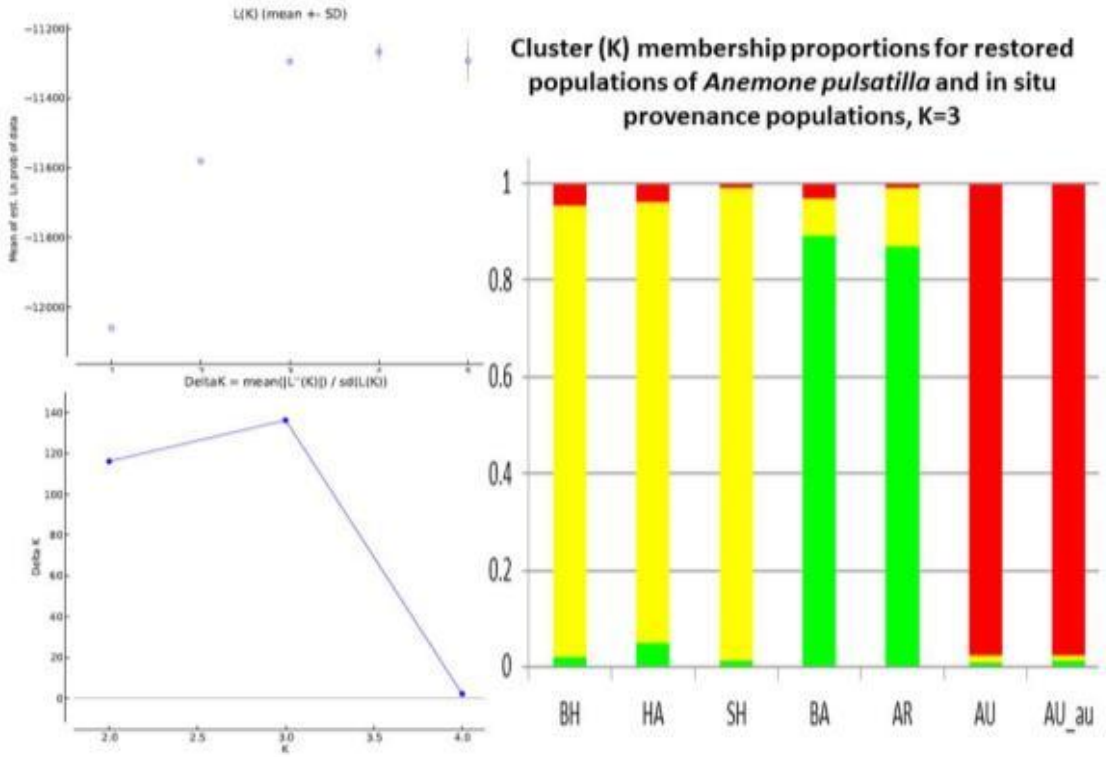


Figure 14. CLUMPP averaged STUCTURE output for restored and in situ wild provenance population of *Anemone pulsatilla* L., STRUCTURE project run for K=1 to K=5 (20 iterations each), parameter set: admixed (ancestry), correlated allele frequencies, 100,000 burin period iterations and 30,000 Monte Carlo sampling reps, graphs for L(K) and Delta K likelihood probability generated by STRUCTURE Harvester, K=3 identified as the cluster (K) number with the greatest L(K) and Delta K likelihood value.

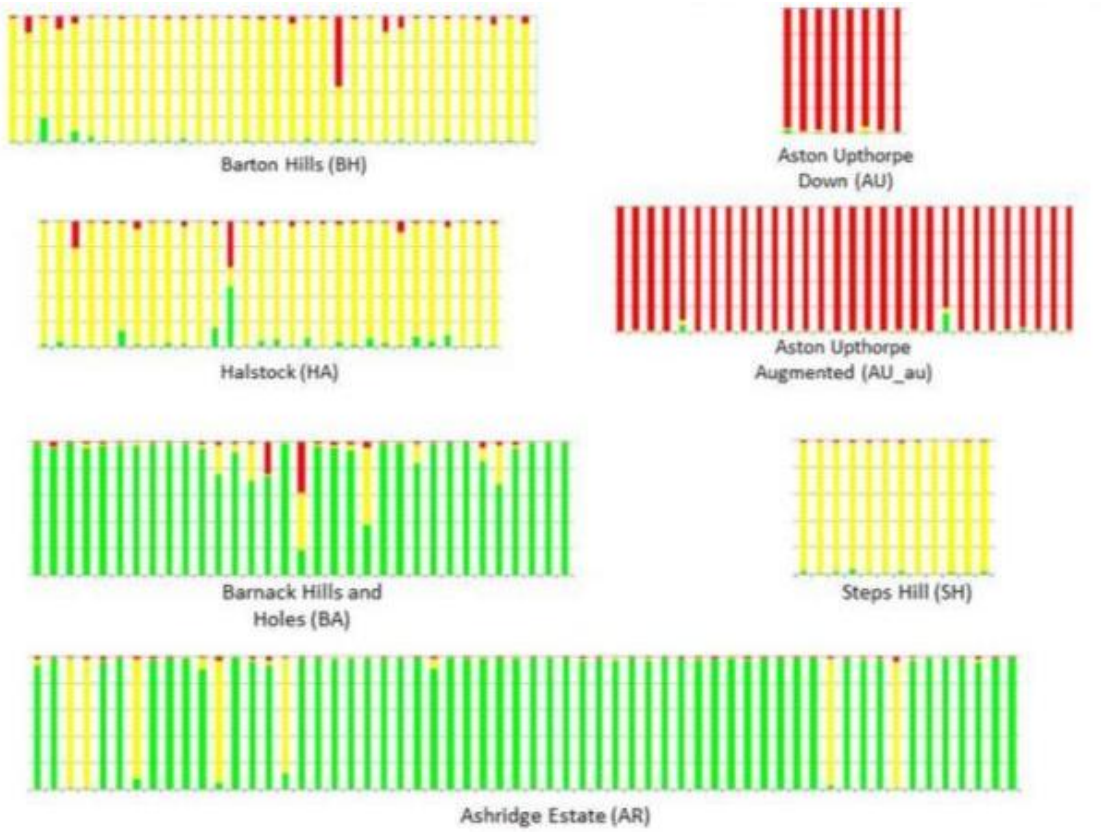


Figure 15. CLUMPP averaged STRUCTURE output of cluster membership proportions (Q) for restoration trial populations of *A. pulsatilla* and their wild provenance populations at K=3, see figure 10 for STRUCTURE Project parameters

4.5 Population Genetic Structuring of Ex Situ Regenerated Populations

Populations WP(BA)11, WP(BA)12, WP(BA)13, JL(BA)13, and WP(BA)14 represent ex situ living, nursery maintained, *A. pulsatilla* populations that are one generation (F1) removed from the wild provenance population Barnack Hills and Holes (BA). Each population has been regenerated ex situ from seed sourced from the Barnack Hills and Holes (BA) seed accession collected in 1999 and maintained at the Millennium Seed Bank (RBG, WP). Population WP(BA)12_F2 represents an ex situ living, nursery maintained, *A. pulsatilla* population that is two generations (F2) removed from the wild provenance population Barnack Hills and Holes (BA). This population has been regenerated ex situ from seed sourced from the ex situ maintained living population WP(BA)12. Population JL(SH)13 represents an ex situ living, nursery maintained, *A. pulsatilla* population that is one

generation removed (F1) from the wild provenance population Steps Hill (SH). This population has been regenerated from seed collected from SH in 2012 and subsequently maintained within a maternal line (i.e. seeds labelled to identify maternal plant) seed accession at the Millennium Seed Bank (RBG, WP). The JL(SH)13 trial was established with equitable representation of each maternal line seed accession.

STRUCTURE Harvester analysis of the STRUCTURE project for ex situ regenerated populations of *A. pulsatilla* and their wild provenance populations calculates a steep decline in likelihood of K values after K=2, observed for both the graph of L(K) and Delta K (figure 16). From population Q bar plots of CLUMPP averaged STRUCTURE estimated cluster membership proportions at K=2 it can be observed that all ex situ regenerated populations are partitioned to the genetic cluster occupied by their respective wild provenance population.

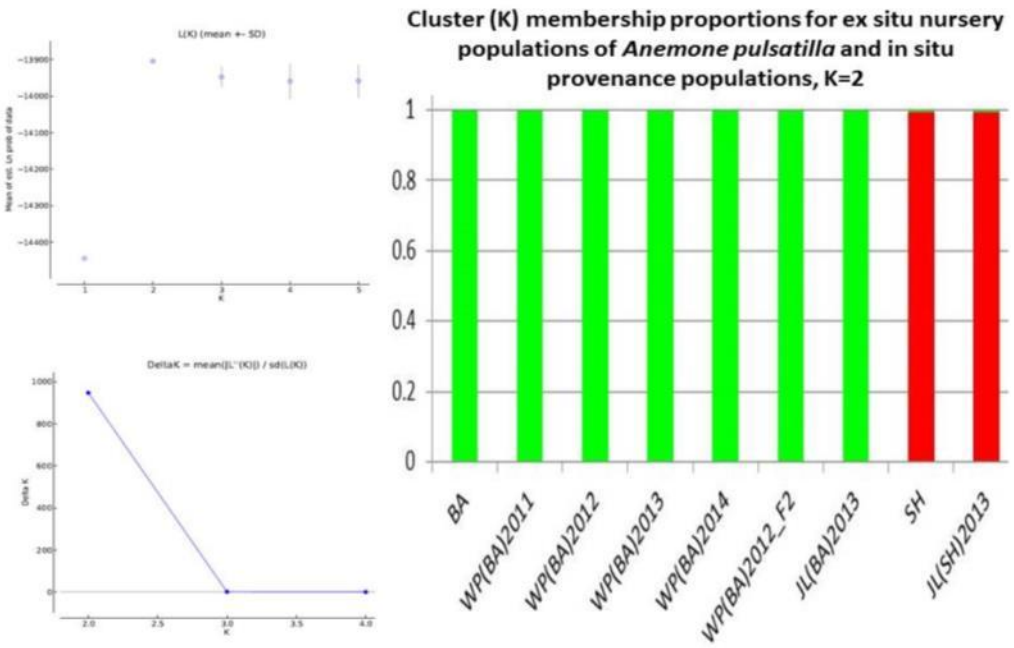


Figure 16. CLUMPP averaged STUCTURE output for ex situ nursery and in situ wild provenance population of *Anemone pulsatilla*, L. STRUCTURE project run for K=1 to K= 5 (20 iterations each), parameter set: admixed (ancestry), correlated allele frequencies, 100,000 burin period iterations and 30,000 Monte Carlo sampling reps, graphs for L(K) and Delta K likelihood probability generated by STRUCTURE Harvester, K=2 identified as the cluster (K) number with the greatest L(K) and Delta K likelihood value.

4.6 Within Population Genetic Diversity of Ex situ and Restored Populations

Allelic diversity counts (loci polymorphism), i.e. the diversity of alleles recorded at each microsatellite loci across all sampled genotypes within a population (calculated by Polysat in R v3.0.2 console), provide a quantified comparison of within population genetic diversity among ex situ regenerated/restoration trial, and wild provenance populations (figure 17). Unique microsatellite alleles (i.e. alleles that occur in just one population) are recorded from raw microsatellite data for each individual at each microsatellite loci (appendix 5).

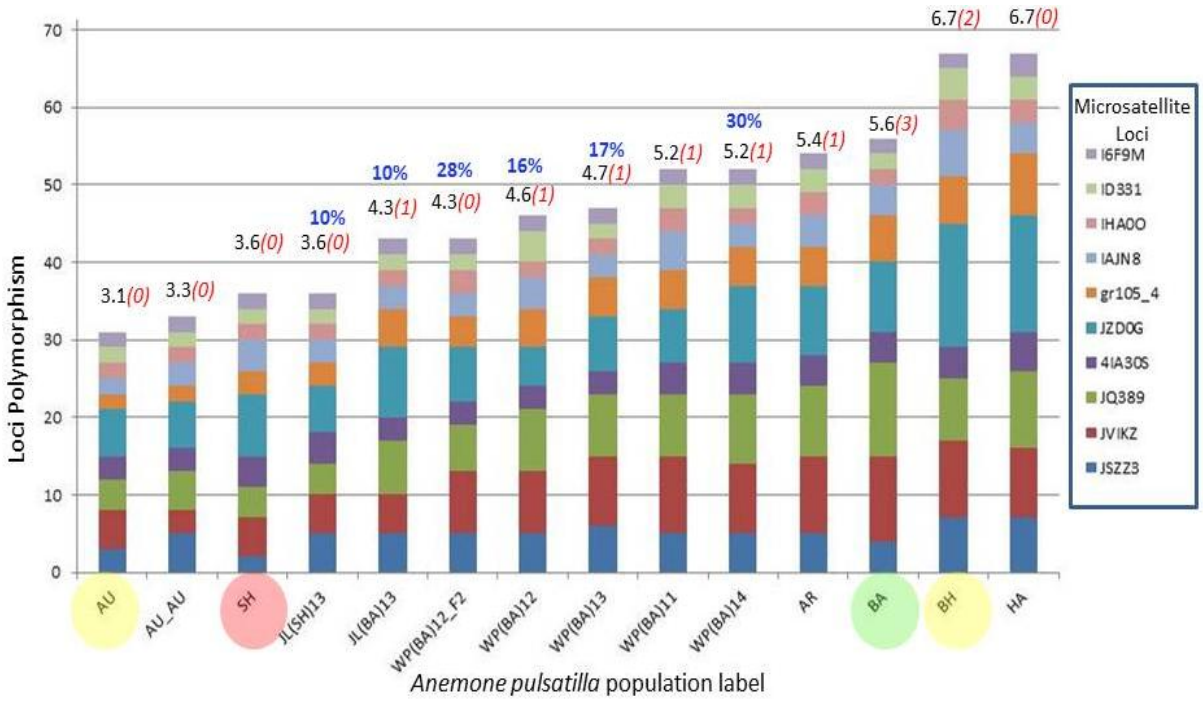


Figure 17. Allelic diversity counts calculated by Polysat in R for 10 microsatellite loci across ex situ regenerated, in situ restored populations of *Anemone pulsatilla* and their wild provenance populations, mean loci polymorphism (averaged across all 10 loci) recorded for each population in numbers above allele count bars with number of unique alleles recorded in brackets, where values are available percentage survivorship, from seed, is recorded for ex situ regenerated, nursery maintained, populations in blue text, along the x axis in situ (wild provenance) populations are coded with the colour of the STRUCTURE estimated cluster membership at K=5 . See table 6 for details on the provenance of restored and regenerated populations.

Mean loci polymorphism counts (i.e. population allelic diversity count/number of microsatellite loci) recorded for restoration trial populations HA and AU_au are comparable to their wild provenance populations (BH and AU respectively). The mean loci polymorphism count recorded for admixed ancestry restoration trial population AR is comparable to the wild provenance population BA and greater than wild provenance population SH.

Of the ex situ regenerated populations, mean loci polymorphism count for the F1, maternal line maintained, population JL(SH)13 is comparable to the wild provenance population (SH). Allelic capture for JL(SH)13 is therefore estimated to approach 100% of natural within population genetic diversity, despite a low survivorship from seed of 10%. Mean loci polymorphism count for F1 populations of BA provenance (WP(BA)11, WP(BA)12, WP(BA)13, JL(BA)13, and WP(BA)14) is recorded as 4.8 alleles/microsatellite loci, fewer than is observed for the wild provenance population. Mean allelic capture for F1 BA provenance ex situ regenerated populations is therefore estimated to be 86% of natural within population genetic diversity. A consistent trend can be observed among F1 BA provenance ex situ regenerated populations for mean loci polymorphism, and therefore percentage allelic capture, to decline in association with survivorship percentage (ranging from 10% to 30% of viable seed). For the F2 ex situ regenerated BA provenance population (WP(BA)12_F2), mean loci polymorphism count is recorded as 4.3 alleles/microsatellite loci, fewer than the mean loci polymorphism count recorded across F1 regenerated BA provenance population. Whilst WP(BA)12_F2 recorded a relatively high survival percentage (28%), the mean loci polymorphism count is equal to F1 BA provenance population recording the lowest survivorship percentage of 10% (JL(BA)13).

A consistent trend can be observed for a decline in unique population allele count among wild provenance and ex situ regenerated/restoration trial populations. The two unique population alleles recorded for BH are not maintained within the restoration trial HA population. Of the three unique BA population alleles, one unique BA population allele is recorded as maintained within the restoration trial AR population and across all first generation (F1) ex situ regenerated populations of BA provenance. No unique BA population allele is recorded as maintained within the second generation ex situ regenerated population of BA provenance. No unique population alleles are recorded for the SH wild provenance population or F1 ex situ regenerated JL(SH)13 population.

5. Discussion

5.1 Research Question 1:

How has range fragmentation, population decline, and restoration intervention influenced the population genetic structure of *A. pulsatilla* throughout the species' extant UK range?

With the central aim to provide an evidence base for complimentary in situ and ex situ gene conservation strategies for *A. pulsatilla,* this research project aims to address two key research questions, the first stated above queries the selection factors influencing the species' UK population genetic structure.

In order to answer research question 1, a sampling strategy was designed to capture a representative sample of the genetic variability of each extant population defined by Walker and Pinches (2011) national survey of *A. pulsatilla.* In total, 331 individuals were sampled and genotyped (at 10 microsatellite loci) across 16 in situ populations. No individuals were located for sampling at three of the 19 in situ sites identified by Walker and Pinches (2011). The 16 sampled in situ populations vary in size from >75, 000 to one individual (Walker & Pinches 2011) and are dispersed throughout the UK range of *A. pulsatilla* in regions which vary in extent of fragmentation (with the greatest population loss and decline observed in the south, west and north east of the species extant UK range). These 16 sampled sites also include two sites (MD and AN) subject to casual restoration intervention (defined as sites where propagules of unverified genetic origin are suspected to have been introduced). Also sampled are 90 individuals across three sites subject to a formal restoration intervention (i.e. introduced propagules of verified genetic origin no more than one generation removed from the native *in situ* source population). At one of

these formal restoration sites, AU_au (a population augmentation), all introduced propagules are sourced from the local native (AU) population (30 individuals samples). At a second site, AR (an introduction) a proportion of individuals are sourced from the local native population (SH) and a larger proportion sourced from a site outside of the local population (BA). At a third site in the southern region of the species UK range, HA (an introduction), propagules originate from a single native population located in the eastern region of the species' UK range (BH). The UK distribution of *A. pulsatilla* therefore provides a natural experiment to test the following hypotheses regarding selection factors influencing population genetic structure of a vulnerable plant species:

5.11 Hypothesis 1(a):

The geographical pattern of range fragmentation across the UK range of *A. pulsatilla* is reflected in the spatial structuring of among population genetic variation.

Population genetic analyses applied to elucidate population genetic structure across *A. pulsatilla* in situ UK distribution included:)a) analyses of genetic distance among genotypes (PCA and PCoA performed using multivariate statistical packages in R v3.0.2 console); (b) quantification of genetic differentiation among populations (F_{ST} values calculated using Polysat in R); 3) Global Spatial Auto-Correlation analysis of the statistical strength of an association between genetic and geographic distance (performed using PopGenReport in R, and; (c) cluster based modelling approaches (performed using STRUCTURE v.2.3.4, STRUCTURE Harvester, and CLUMPP v.1.2.2).

Positive spatial auto-correlation observed for natural in situ populations of *A. pulsatilla* in the UK provides statistical support for a dependent association between genetic and geographic distance, i.e. the spatial structuring of population genetic variation across

the fragmented in situ UK range of *A. pulsatilla* (figure 8). However, some caution should be taken in interpreting this spatial auto correlation as entirely dependent on fragmentation, as distance is generally associated with increasing biological differentiation across a species' range.

Graphical representations of genotypic variation among in situ *A. pulsatilla* populations (i.e. PCA and PCoA) support an interpretation that spatial structuring of population genetic variation reflects broadly geographical patterns of population distribution and degree of range fragmentation (i.e. in support of hypothesis 1a). For example, along the 1st axis of PCA and PCoA graphs a distinct trend is observed for structuring of the four western distributed populations into two distinct genetic clusters (distributed at the extreme left of the 1st PCA and PCoA axis) whereas no distinct genetic structuring is observed for eastern region populations (distributed at the extreme right of the 1st PCA/PCoA axis). This regional variation in the extent of population genetic structure reflects regional variation in the extent of historical population loss and decline (i.e. range fragmentation). For example, a high rate of population loss and decline has been recorded within the western region of *A. pulsatilla* UK range (with three of four extant populations numbering c. 500 or fewer individuals) whilst eastern region populations represent the centre of extant UK population density (supporting four of the five largest UK populations), figure 3. A high level of population loss and decline has also been recorded in the southern and north eastern regions of the species' UK range. The north eastern distributed population BA is distinctly clustered from other UK populations on the PCoA graph along the 2nd axis. Therefore, four discretely variable population genetic clusters (reflecting the geographical patterns of population distribution and extent of range fragmentation) emerge

from PCoA analysis (which excludes populations subject to formal or informal restoration intervention).

STRUCTURE output from K=2 to K=5, when mapped to the geographic locations of extant *A. pulsatilla* populations (figure 11), also supports hypothesis 1a that geographic patterns of range fragmentation across the UK range of *A. pulsatilla* results in spatial structuring of among population genetic variation. In structure analysis inclusive of all 16 *in situ* populations (including the casually restored MD and AN populations) K=5 emerges as the most supported cluster value to explain the greatest amount of among population genotypic variation (figure 5). With one distinct population genetic cluster defined by AN (i.e. inclusive of individuals of unverifiable genetic origin) the CLUMPP averaged STRUCTURE output for K=5 (figures 10 and 11) supports PCoA analysis of four discretely variable natural population genetic clusters which reflect geographical patterns population distribution and historical range fragmentation (i.e. an eastern population genetic cluster, two sub-structured western population genetic clusters, and a north eastern population genetic cluster defined predominately by BA).

Pairwise F_{ST} values, which quantify genotypic variation among populations, provide additional statistical support for hypothesis 1a (table 9). Near great differentiation (F_{ST} = 0.125 to 0.149) calculated among western region populations HR and BD and the north eastern region population BA reflects the distinct genotypic variation observed among these population clusters in PCoA and STRUCTURE analyses. In addition, among eastern region populations genetic differentiation (pairwise F_{ST} values) is calculated to be low, i.e. 0.03 or less (appendix 3), reflecting the lack of distinct genotypic variation observed among these populations within PCA/PCoA and STRUCTURE analysis.

Pairwise F_{ST} values can therefore be interpreted to support observations drawn from multivariate (i.e. PCA and PCoa) and STRUCTURE analysis that spatial trends emerging from genotypic variation among native in situ *A. pulsatilla* UK populations reflect geographical patterns of range fragmentation

Emergence of population genetic structure within highly fragmented regions of a species' range, as observed for natural populations of *A. pulsatilla* within this study, can be explained by conservation genetic theory to be a result of the random (stochastic) population genetic processes (such as founder effect and genetic drift) to which small and isolated populations are vulnerable (Young *et al.* 1996; Sherwin & Moritz 2000; Frankham *et al.* 2002; Allendorf *et al.* 2013). The positive spatial auto-correlation observed for natural in situ populations of *A. pulsatilla* can be interpreted to support a scenario of genetic drift driving the emergence of population genetic structure, as an artefact of declining population size and loss of gene flow across the species' fragmented UK range (Smouse & Peakall 1999). Structuring of genetic variation among fragmented *A. pulsatilla* populations can be predicted to increase the risk that rare, locally adaptive, alleles and/or genotypes are lost from populations via random drift of allelic frequencies without the opportunity for re-introduction via inter-population gene flow (Young *et al.* 1996; Sherwin & Moritz 2000; Frankham *et al.* 2002; Allendorf *et al.* 2013). Restoration of gene flow among populations can be predicted to reduce the risk of rare allele/genotype loss through random population genetic processes. However, an understanding of environmental variation among populations should be applied to inform the process of restoring inter-population gene flow, to reduce the risk of out breeding depression (i.e. swamping of a locally adapted population with genotypes which infer reduced fitness to the local environment).

5.12 Hypothesis 1(b):

Declining *A. pulsatilla* population size is associated with declining representation of the species' natural genetic variation.

Allelic diversity counts (loci polymorphism), i.e. the total number of different alleles recorded at each microsatellite loci across all sampled genotypes within a population (calculated by Polysat in R v3.0.2 console) provide a means to quantify within population allelic richness (i.e. mean loci polymorphism). Unique microsatellite alleles (i.e. private alleles that occur in just one population) are recorded from raw microsatellite data for each individual at each microsatellite loci (appendix 4).

Wide variation of within population genetic diversity counts can be observed across the UK range of *A. pulsatilla* (figure 13). Of the 14 natural in situ populations sampled across the species native UK range (i.e. excluding AN and MD which include individuals of unverified genetic origin) eight of the ten populations composed of <1000 individuals (Walker & Pinches 2011) fall in the lower range of allelic richness (averaging <5 alleles/loci). The two populations (RC and DD) composed of <1000 individuals which fall in the upper range of loci polymorphism (i.e. averaging > 5 alleles/loci) are both located within the eastern region of the species' range where fragmentation has occurred to a lesser extent than elsewhere. It can be suggested therefore that the greater representation of species' genetic variation in RC and DD than other populations numbering <1000 individuals is a result of RC and DD experiencing restricted inter-population gene flow over a shorter time period than other smaller populations (RC and DD are therefore at a lower risk of chance allele loss and genetic diversity decline).

Notably, the two largest *A. pulsatilla* UK populations (TH composed of 60, 000 individuals and BW composed of 75, 000 individuals) occupy two extremes of allelic richness counts within the upper region of population genetic diversity (i.e. averaging >5 alleles/loci). BW, averaging 5.1 alleles/loci with no private alleles, is located within the fragmented western region of the species' extant UK range whilst TH, averaging 7.9 alleles/loci with 8 private alleles (the highest of all in situ populations) is located at the centre of the species' extant UK distribution where population loss and decline has been lowest.

It should also be noted that of the eight populations falling within the lower range of population genetic diversity, six record sample sizes of <30, sampling was restricted by the number of flowering individuals identified on the day of the sampling visit. Within the upper range of population genetic diversity, four populations record samples sizes of >30 (figure 13). A sample size of 30 is supported as providing a reasonable probability of obtaining a representation of population genetic diversity (i.e. >95% allelic capture) within natural biological populations (Luikart *et al.* 1998).

Therefore, whilst is can be inferred that there is broad support for declining population size resulting in declining representation of the species' natural genetic variation, a caveat should be introduced that sample size, and local range fragmentation (i.e. duration of reproductive isolation) is also a likely influential factor on population genetic diversity. Rarefaction analysis can be applied to provide statistical analysis for the influence of sample size on representation of population genetic diversity (i.e. by removing individuals one by one from sampled populations and analysing the impact genetic diversity). However, of the statistical packages available for rarefaction analysis (i.e. vegan in R, FSTAT, and Heirfstat)

there does not appear to be an open source package available which can interpret polyploid data files.

5.13 Hypothesis 1(c):

The introduction of propagules of unverifiable genetic origin to the species' UK range will result in structuring of genetic variation.

Five populations within the UK range of *A. pulsatilla* subject to some form of restoration intervention have been sampled for genotypic variation to test the above hypothesis (nature of restoration intervention is described briefly in section 5.1). Two of these restoration intervention populations (MD and AN) are included within the 19 extant UK populations of *A. pulsatilla* recoded by Walker and Pinches (2011) and are therefore also included within the 16 in situ populations sampled for this study. MD falls outside the historical UK range of *A. pulsatilla* and was first recoded it 2011 and is therefore assumed to originate from an unrecorded (i.e. causal) restoration intervention. AN has been recorded to be the subject of a restoration intervention by Lincolnshire Wildlife Trust (22 introduced and six native propagules were recorded by Walker and Pinches (2011)) although the genetic origin of the introductions are unverified. When AN was sampled to provide genetic material for this study, the samples supplied were not identified as of native or introduced origin. Hypothesis 1c predicts that introduction of individuals of unverified local origin will introduce genetic structure to the UK range of *A. pulsatilla*.

From STRUCTURE analyses of population genetic variation across the UK range of *A. pulsatilla* (i.e. inclusive of 16 of the 19 populations described by Walker and Pinches (2011)) AN emerges as a discrete genetic cluster at K=2 (figures 10 & 11). AN remains as a distinct population genetic cluster at K=3, K=4, and K=5. Genotypic variation represented by AN is

therefore estimated by STRUCTURE to diverge from population allelic frequencies represented by all other extant UK populations (figures 10 and 11), supporting hypothesis 1c. Further support for hypothesis 1c is provided by F_{ST} analysis in which AN is calculated to be greatly genetically differentiation (Pairwise F_{ST} between 0.15 and 0.25) from populations representing all four of the natural population genetic clusters (defined by STRUCTURE and PCoA) (table 9). In STRUCTURE analysis of among population genotypic variation MD clusters with the western region population genetic cluster defined by BW, HG and SH at K=5 (the most strongly supported K value for the UK range of *A. pulsatilla*). MD therefore does not appear to diverge significantly from allelic frequencies represented in natural UK populations and may therefore be assumed to share a genetic origin with in situ populations occupying the shared genetic cluster (most likely the largest UK population of BW).

From STRUCURE analysis of individual genotypic variation across the UK rage of *A. pulsatilla,* genetic cluster membership proportions are generally consistent among individuals within populations (figure 12). For AN, however, six genotypes vary distinctly from the other 14 genotypes in cluster membership proportions. The six distinct AN genotypes which do not cluster with the other 14, divergent, AN genotypes can be observed to share half or more of their genotypic variation with other natural population genetic clusters. The discretely clustered 14 genotypes of AN share a negligible proportion of genotypic variation with other population genetic clusters across the species' UK range. It can be predicted with relative confidence that the six AN genotypes which cluster predominately with other natural population genetic clusters represent the six native individuals recorded at AN in 2011. Therefore, the significant divergence of AN population allelic frequencies from other UK populations can be estimated to result from the casual

restoration intervention. The six assumed native AN genotypes do share a proportion of admixed ancestry with the 14 assumed introduced genotypes. It is plausible, therefore, that introduced propagules are multiple generations removed from the natural AN population (i.e. multiple generations of ex situ regeneration) resulting in significant drift from natural allelic frequencies. In support of this conclusion is the observation from raw genotypic data (Appendix 4) that the four private alleles represented in AN (figure 13) correspond to the six admixed (assumed native) genotypes (i.e. STRUCTURE analysis allows for identification of individual genotypes). Therefore, the small number of assumed native genotypes at AN represents a great proportion of the UK's native population genetic diversity (average number of private alleles among the 16 in situ populations is 2.1 per population) which is under represented by the casual restoration intervention.

Three populations are subject to restoration intervention of verifiable genetic origin (i.e. introduced propagules no more than one generation removed the native UK population/s of origin). Two restoration populations (AU_au and HA) originate from a single source in situ population within the UK range (AU and BH respectively). These restoration populations share equivalent genetic cluster membership proportions with their respective source populations, observed in the STRUCURE analysis of population genetic variation among source and restored populations (figure 14). The restored population AR is subject to restoration intervention via the introduction of propagules from two in situ UK populations, the local SH population and a greater proportion from the more distant BA population. The larger and more diverse source population BA (figure 13) is greatly over represented in STUCTURE analysis of population genetic variation among source and

restoration populations (figure 14). BA genotypic variation virtually obscures the population genetic signal of differentiated SH genotypes (figure 15).

Observations of this study suggest that restoration intervention via introduction of propagules of verifiable native genetic origin, no more than one generation removed from in situ origin population, allow estimation of relative confidence that restored populations are at a low risk of genetic divergence from the in situ populations. Caution should be applied to introducing propagules of variable genetic origin due to the risk of population genetic sub-structuring emerging among individual genotypes (see figure 15). Where local populations are small and therefore likely of reduced genetic diversity, it is recommended that propagules originating from larger and more diverse populations occupying the shared genetic cluster are sought as appropriate source populations. It can also be noted that HA and and AU_au capture a great proportion of the genetic diversity (quantified as average allelic diversity/locus) represented by the natural source populations of BH and AU respectively (figure 17). AR (two genetic origins for introduced propagules) captures an equivalent level of genetic diversity as represented in the source population BA (average of 5.4 and 5.6 alleles/locus respectively) which is more diverse than the local source population AU (averaging 3.3 alleles/locus). The informal population restoration intervention at AN however fails to capture the unique genetic variability represented in native genotypes, highlighting the importance of verifying genetic origin of introduced propagules in order to maximise opportunities for maintaining a species' genetic integrity.

5.2 Research Question 2:

How well do ex situ conservation measures for *A. pulsatilla* represent the species' natural genetic variability?

A pilot study was designed under research question 2 to sampling genetic diversity of seven ex situ regenerated *A. pulsatilla* accessions, allowing for an initial exploration of selection factors influencing ex situ representation of natural genetic variation.

5.21 Hypothesis 2

Ex situ conservation practices for the establishment and maintenance of accessions can impose high selection pressures on genetic diversity resulting in under-representation of natural genetic variation.

Ex situ conservation practices which can be predicted to limit genetic diversity of accessions, resulting in under-representation of a species' natural variability, include: (a) low sampling effort across a species' in situ range; (b) increasing number of generations ex situ accessions are removed from in situ source populations, (c) increasing rate of attrition of regenerated *ex situ* accessions (i.e. survival rate of seed to established plant), and: 4) low effective population size (i.e. proportion of parental genotypes contributing to the next generation).

The existing, long term, ex situ gene conservation strategy for *A. pulsatilla* represents limited sampling of the species' in situ UK range, inclusive of seedbank accessions sampled from two populations (BA and SH). The evidence base gathered under research questions 2 of this study supports a scenario of the partitioning of genetic variation among four discrete natural population genetic clusters across the species' UK range.

Therefore, it can be predicted that the species' natural genetic variability is currently under-represented ex situ as a result of low sampling effort. At a minimum, a representative ex situ gene conservation strategy for *A. pulsatilla* should aim to maintain accessions sampled from the most diverse populations of each of the four main genetic clusters identified by population genetic analyses (figures 7 and 13).

The establishment of banked seed accessions can provide a safeguard for vulnerable species' natural genetic diversity. As a tool for in situ restoration ex situ gene conservation strategies must aim to maintain genetic representativeness through practices such as regeneration of seed accessions to provide propagules for reintroduction. Seven regenerated *A. pulsatilla* accessions sampled for this study vary in ex situ factors which can be predicted to influence selection pressure on genetic diversity, such as: (a) number of generations the ex situ regenerated accession is removed from in situ source population, and; (b) survival rate of seed to established plant, and; (c) strategies applied to maintain a high effective population size. Six of the regenerated accessions are one generation removed from the in situ source population whilst one (WP(BA)12_F2) is two generations removed (the parental accession being WP(BA)12). Survivorship (percentage of seed surviving to established plant) varies across the seven accessions from 10% to 30%. One of the regenerated accessions is maintained in maternal lines as a strategy to maintain a high effective population size (JL(SH)13). Therefore, these regenerated provide an opportunity to explore the influence of a number of ex situ selection factors on representation of natural genetic variation.

Under the existing gene conservation strategy for *A. pulsatilla,* the regenerated accessions described are excluded from in situ restoration as it is hypothesised that <50%

survivorship will result in under-representation of natural genetic diversity. Second generation (or greater) regenerated ex situ accessions are also exclude from in situ restoration under the existing gene conservation strategy for *A. pulsatilla* as it is hypothesised that greater than one generation removal from the in situ source population will result in under-representation of natural genetic diversity. There is however, no evidence base for these hypotheses. The existing gene conservation strategy places a limitation on the application of ex situ conservation as a tool for in situ restoration of *A. pulsatilla* genetic diversity due to the typically low rate of recruitment from seed recorded for the species' in situ and ex situ. However, this study can provide some evidence base for development of an effective gene conservation strategy which makes efficient use of available ex situ resources for restoration whilst minimising the risk of under-representation of natural genetic variation among restored populations.

A genetically representative *ex situ* accession will represent 95% genetic variability (i.e. 95% allelic capture) of the source, in situ, population. Of the seven regenerated accessions sampled one first generation accession (JL(SH)13) was observed to maintain genetic diversity (i.e. allelic richness) counts equitable to the parental population (SH), i.e. c. 100% allelic capture (figure 17). Survivorship was low for this regenerated accession (10% of seed converted to established plant) and so it could have been predicted that this accession would under-represent genetic variation of the in situ source population. However, JL(SH)13 is the only regenerated accession among those sampled for this study to have bene maintained in maternal lines, i.e. the maternity of in situ collected seed is recorded to maximise opportunity for parental genotypes to be equitably represented in succeeding generations). Therefore, observations of this study suggest that applying strategies to

maintain high effective population size for regeneration of ex situ accessions is effective in mitigating the limiting effect of low survivorship on genetic representativeness. As a comparison, the ex situ accessions (JL(BA)13) regenerated from a mixed seed lot and recording the equivalent survivorship of 10% achieved 77% allelic capture, i.e. under-representation of natural genetic diversity.

A general trend can observed among *A. pulsatilla* ex situ accessions regenerated from mixed seed lots for percentage allelic capture (i.e. representativeness of source population genetic diversity) to increase with survivorship (figure 17). At 30% survivorship, regenerated accession WP(BA)14 achieves 93% allelic capture, close to the 95% threshold for true genetic representativeness. The second generation WP(BA)12_F2 ex situ accession achieves a relatively high rate of survivorship (28%), however allelic capture at c. 77% is equivalent to the first generation accession with the lowest survivorship rate of 10% (JL(BA)13). Allelic capture for the parental accession of WP(BA)12_F2 (i.e. WP(BA)12) is c. 83%. Therefore, as predicted, increasing the number of generations an ex situ accessions is removed from the source in situ population resulted in increasing genetic diversity loss and under-representation of natural genetic variation. This initial pilot study supports the prediction that the following factors will result in ex situ genetic diversity loss for regenerated accessions: (a) low effective population size; (b) poor survival, and; (c) more than one generation removal from an in situ source population. A fully replicated trial (and statistical analyses of allelic richness) is required to provide scientific support for these initial observations and therefore provide a robust evidence base to inform ex situ gene conservation strategy.

STRUCTURE analyses of genetic variation among *A. pulsatilla* regenerated accessions and in situ source populations reveals no significant allelic frequency divergence of ex situ and in situ genotypes (figure 16). Further experimentation would be required to test the hypothesis that ex situ conservation practices will result in genetic divergence of regenerated accessions many generations removed from in situ parental populations.

6. Conclusions

6.1 Research Question 1

The population genetic structure of the vulnerable UK plant species *Anemone pulsatilla* reflects geographic patterns of historical range fragmentation and the influence of population decline and restoration intervention. Positive spatial auto-correlation of natural in situ populations of *A. pulsatilla* lends support to a scenario for genetic drift (i.e. random drift of allelic frequencies) driving the emergence of population genetic structure as a consequence of fragmentation. Multivariate and STRUCTURE analysis estimates the partitioning of genetic variation among four natural population genetic clusters (broadly defined by geographical regions of the species' range) and a fifth, highly differentiated, genetic cluster defined by introduced genotypes of unverifiable genetic origin to the casually augmented AN population.

The key aim of this research study is to provide an evidence base to support recommendations for an integrated in situ and ex situ gene conservation strategy which can support species' recovery in the UK. It is recommended, therefore, that restoration intervention is focused on regions of the species range which have experienced the greatest range fragmentation, population decline, and structuring of genetic variation, (western, north eastern, and southern regions). Restoration intervention which supports gene conservation should be guided by conservation genetic principles, such as: 1) increasing population size through management/introduction in order to reduce vulnerability to random selection pressures; 2) sourcing introduced genotypes from genetically diverse populations which share similar allelic frequencies (i.e. no significant among population genetic variation), and; 3) restoring gene flow among neighbouring population fragments to

facilitate exchange of locally adaptive genotypic diversity. Recommendations for further study to strength the evidence base underlying species' recovery and in situ gene conservation strategies for *A. pulsatilla* is an analyses of the variable natural selection pressures acting on extant populations. This analysis can be applied to inform gene flow restoration intervention which supports exchange adaptive genetic variation and limits the risk of out breeding depression.

6.2 Research Question 2

The existing ex situ gene conservation strategy for *A. pulsatilla* can be predicted to under-represent the species' natural genetic variability, due to: 1) limited sampling effort inclusive of seed accessions collected from just two in situ populations, and; 2) partitioning of genetic variability among distinct genetic clusters across the species' UK range.

Recommendations of this study for development of a representative ex situ gene conservation strategy is for the establishment of banked seed accessions (as the most efficient means for the long-term safeguard of viable plant material) sampled from: 1) TH (the most diverse of all UK populations) to represent the eastern region in situ population genetic cluster; 2) BA, the largest and most diverse population of the north eastern region genetic cluster; 3) BW, the largest and most diverse of one of the western region population genetic cluster, and; 4) both of the small populations HR and BD which define the second western region genetic cluster. It is also recommended that an ex situ accession is established to represent native AN genotypic variation. This study has demonstrated that the small number of native AN genotypes (outnumbered by introduced individuals of unverified genetic origin) capture unique *A. pulsatilla* genetic variation (in the form of private alleles) which are not represented by the introduced genotypes at this site.

Observations from a pilot study of ex situ selection pressures acting on regenerated *A. pulsatilla* accessions (i.e. living ex situ collections regenerated from banked seed) support the prediction that the following factors can increase the risk of genetic diversity loss: 1) increasing number of generations ex situ accessions are removed from in situ source populations; 2) increasing rate of attrition of regenerated *ex situ* accessions, and;: 3) a lack of a breeding strategy to support high effective population size.

A key observation of the ex situ gene conservation pilot study is that first generation ex situ regenerated *A. pulsatilla* accessions can be predicted to approach true genetic representativeness of in situ source populations at 30% survival rate. The existing gene conservation strategy for *A. pulsatilla* (operated by the Native Seed Hub, RBG WP) excludes first generation regenerated accessions of <50% survival rate from in situ restoration due to the assumption of a high risk of under-representation of natural genetic variation. There is no evidence base for the 50% survival threshold and therefore it is recommended that, on the basis of this study, the threshold survival rate for inclusion of *A. pulsatilla* ex situ regenerated accessions within species in situ restoration programmes is reduced to 30%. Due to the poor seed recruitment rate generally recorded for *A. pulsatilla,* ex situ and in situ, a 30% survival rate will facilitate a more efficient use of genetically representative ex situ conservation resources as a tool for in situ species recovery.

A fully replicated ex situ gene conservation trial (and statistical analyses of allelic richness) is required to provide scientific support for these initial observations and to provide a strong evidence base to inform ex situ gene conservation strategy for *A. pulsatilla*.

6.3 Proposal for further study

Inference of a fitness cost for populations experiencing declining neutral genetic diversity (i.e. reduced microsatellite loci polymorphism) must be attached to the caveat that no consistent trend has emerged from published studies for the covariance of adaptive and neutral genetic variation among populations. The trend for declining allelic richness observed for small in situ populations and select ex situ regenerated populations in this study provides an opportunity to test the hypothesis that declining neutral genetic diversity is associated with fitness decline (i.e. by identifying co-varying phenotypic traits). The phenotypic trait of seed weight has been observed to be associated with neutral population genetic diversity of *A. pulsatilla* among German in situ populations (Henson *et al.* 2005).

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8. Appendices

Appendix 1: Protocol for DNA mini-extractions using the Mixer Mill MM301

- Pre-cool the adapters and lids (white plastic) in -20°C freezer for at least 30 minutes, up to a few hours.

- Use approximately 20-60mg of leaf tissue (usually a piece of dry leaf as large as a third or a half of your fingernail). For very leathery leaves you may want to cut or break it into smaller pieces to improve the result. You might also be able to start with more material, depending on your species and tissue. Don't overload with tissue as the buffers of your DNA extraction protocol will not function efficiently.

- After tissue grinding it is important to transfer the ground material into buffer quickly, therefore you need to have the buffers ready. Calculate the amount of 2X CTAB buffer and β-mercaptoethanol that you need for all your samples (proportion of 10ml of 2X CTAB for 40µl of β-mercaptoethanol – about 750µl per sample) and place it in a large tube (e.g. 50ml extraction tube). Preheat the buffer in a 65°C water bath.

- Assemble everything for grinding. Checklist: small amount of sand, 1 bead and leaf material inside each 2ml Eppendorf. Place the Eppendorf tubes inside adapters.

Place the lids on adapters. Insert this into mixer mill. Press down the metal knob then turn handle until tight (hear it clicking). Check with your hands whether everything is sitting tight. ALWAYS use 2 adapters. Close hood on mixer mill.

- Set desired time (minutes). Start at low speed for a few seconds and check if it runs smoothly. Then turn up to full speed (frequency '30') to grind.

- Normally 2 minutes grinding is enough. For some difficult specimens you may consider removing adapters from mill, changing orientation of adapters in the mill 180 degrees, then repeating for 1-2 minutes.

- Add 750µl of preheated buffer into the tubes. If you do many samples, always get the freshly ground samples into buffer quickly. Shake the tubes to ensure all of the sample is in buffer.

- Incubate at 65°C for no longer than 20 minutes and shake the tubes halfway through incubation period. If you have many batches, after the 20 minutes get the samples out of the bath and leave them at room temperature.

- Add an equal volume (750µl) of SEVAG (containing chloroform), mixing gently but thoroughly. Extract for up to one hour for slimy or mucilaginous samples by rocking (lie the tubes on their side on the rocker).

- Spin at 8000rpm (9000rpm) for 10 minutes at room temperature. Ideally, but not terribly often, the aqueous (top) phase will be clear and colourless.

- Remove aqueous (top) phase containing DNA and transfer to a new Eppendorf tube. Dispose of SEVAG and plant debris in the SEVAG waste container (do not overfill the waste container above the shoulder). Be careful, you need to keep the metal bead inside the tube.

- Recover the metal bead in each tube after first centrifugation step. Wash with distilled water and return to where you found them. Also return adapters and lids to where you found them.

- You may purify directly this top layer with columns. Alternatively you can precipitate the DNA (this would be especially advisable for AFLPs).

If you want to precipitate your DNA, follow the next steps.

- Add 2X volume of -20°C ethanol (or 2/3s volume of -20°C isopropanol) and mix gently to precipitate DNA. Put in -20°C freezer overnight (leaving them for many hours or up to one day does not seem to be a problem). NOTE: ensure that the sample and ethanol/isopropanol are thoroughly mixed before putting in the freezer.

- Take samples out of the -20°C freezer.

- Spin in a centrifuge at 3000-4000rpm for 5 minutes to collect precipitate. Pour off liquid in a waste container in the fume cupboard. Add 750µl of 70% ethanol. Dislodge the pellet to facilitate “washing” and wash for 5-60 minutes.

- Spin down DNA at 3200-4000rpm for 3 minutes. Pour off liquid and drain upside down for 5-10 minutes (up to a couple of hours) to allow alcohol to evaporate. Be careful not to lose the pellet, sometimes it is better to lie the tube on its side (if your sample is too dirty, you may have to wash twice). You can leave the tubes in the fume cupboard overnight to evaporate the alcohol completely.

- Resuspend your DNA in 125µl of 0.1M TE buffer. When the DNA is dissolved you can purify it with the columns.

Appendix 2: Protocol for "column cleaning" DNA samples using QIAGEN QIAquick Purification Kit

- Add 5 volumes of buffer PB to 1 volume DNA and mix.

- Load the samples into the columns by pipetting, place the columns in the provided 2ml lidless tubes and centrifuge at 13'000rpm for 1-2 minutes.
  The maximum loading volume of the column is 800µl. For sample volumes greater than 800µl simply load the columns again.

- To wash, add 750µl of buffer PE (100ml buffer PE to 400ml 100% ethanol) to each column and centrifuge the columns in the lidless tubes at 13'000rpm for 1-2 minutes.

- Empty the lidless tubes and spin again for 1-2 minutes.
  IMPORTANT: this spin is necessary to remove residual ethanol (buffer PE)

- Place each column into a new 1.5ml microfuge tube.

- Add 100µl of buffer EB (10mM Tris-HCl, pH8.5) to the centre of each column and leave to stand for 30 minutes.

- Centrifuge for 1 minute at 13'000rpm. Buffer can be heated to 65°C in the oven to increase yield.

- Discard any unused buffer.

- Store samples in the fridge.

Appendix 3: Population genetic differentiation (F_{ST} values) among *Anemone pulsatilla* L. in situ UK populations

	BA	L	BW	HG	SV	AU	MD	SH	KH	BH	RC	TH	DD	HR	AN	BD
BA	0.00	0.03	0.09	0.09	0.03	0.08	0.07	0.08	0.08	0.06	0.06	0.06	0.05	0.14	0.13	0.13
L	0.03	0.00	0.02	0.06	0.10	0.09	0.07	0.06	0.02	0.02	0.04	0.02	0.02	0.03	0.04	0.04
BW	0.09	0.02	0.00	0.04	0.06	0.07	0.04	0.06	0.04	0.05	0.04	0.04	0.05	0.05	0.14	0.06
HG	0.09	0.06	0.04	0.00	0.12	0.16	0.08	0.09	0.08	0.06	0.09	0.07	0.07	0.07	0.17	0.07
SV	0.03	0.10	0.06	0.12	0.00	0.10	0.12	0.09	0.03	0.04	0.05	0.04	0.03	0.07	0.08	0.07
AU	0.08	0.09	0.07	0.16	0.10	0.00	0.13	0.11	0.04	0.03	0.04	0.04	0.04	0.10	0.14	0.10
MD	0.07	0.07	0.04	0.08	0.12	0.13	0.00	0.08	0.05	0.04	0.08	0.04	0.05	0.08	0.14	0.09
SH	0.08	0.06	0.06	0.09	0.09	0.11	0.08	0.00	0.05	0.05	0.07	0.06	0.06	0.10	0.16	0.11
KH	0.08	0.02	0.04	0.08	0.03	0.04	0.05	0.05	0.00	0.03	0.02	0.02	0.02	0.09	0.13	0.10
BH	0.06	0.02	0.05	0.06	0.04	0.03	0.04	0.05	0.03	0.00	0.02	0.02	0.03	0.08	0.12	0.09
RC	0.06	0.04	0.04	0.09	0.05	0.04	0.08	0.07	0.02	0.02	0.00	0.02	0.02	0.06	0.11	0.06
TH	0.06	0.02	0.04	0.07	0.04	0.04	0.04	0.06	0.02	0.02	0.02	0.00	0.02	0.08	0.11	0.08
DD	0.05	0.02	0.05	0.07	0.03	0.04	0.05	0.06	0.02	0.03	0.02	0.02	0.00	0.08	0.12	0.08
HR	0.14	0.03	0.05	0.07	0.07	0.10	0.08	0.10	0.09	0.08	0.06	0.08	0.08	0.00	0.16	0.02
AN	0.13	0.04	0.14	0.17	0.08	0.14	0.14	0.16	0.13	0.12	0.11	0.11	0.12	0.16	0.00	0.17
BD	0.13	0.04	0.06	0.07	0.07	0.10	0.09	0.11	0.10	0.09	0.06	0.08	0.08	0.02	0.17	0.00

FST range	Population Differentiation
0 – 0.05	Low
0.05 – 0.15	Moderate
0.15 – 0.25	Great
> 0.25	Very Great

Appendix 4: Raw microsatellite data for in situ UK populations of *Anemone pulsatilla* L.

- Microsatellite alleles recorded for each population under 10 loci
- Microsatellite alleles listed in base pair length order
- Unique population alleles in red text

	JSZZ3	JVIKZ	JQ389	41A3OS	JZD0G	gr105_4	IAJN8	IHA0O	ID331	I6F9M	Unique Alleles
L	205, 229	153, 161, 166	206, 211	160, 167	273, 279, 281, 296	269, 272	130	240, 244	265, 267	240, 243	1:1
AN	197, 202, 205, 208, 231	153, 161, 166, 174, 175, 176, 181, 194, 202	181, 189, 204, 216, 219, 221, 222, 230	155, 160, 167, 173	275, 277, 279, 281, 287, 288, 290, 292, 296, 308	257, 260, 275, 281	111, 130, 132, 134, 136, 138, 141	240, 244, 252	263, 265, 267	240, 243, 246	20:4
SV	205, 208	161, 165, 166, 173, 174, 175, 176, 190	181, 189, 197, 199, 204, 216, 217, 221	155, 160, 167, 173	275, 277, 285, 287, 288, 296, 308	260, 275, 281	111, 113, 132, 136, 138, 141	240, 241, 244, 252	263, 267	240, 243, 246	6:1
BA	202, 205, 208, 211	153, 157, 161, 165, 166, 173, 174, 175, 176, 177, 194	181, 182, 189, 204, 211, 213, 216, 221, 222, 225, 227, 229	155, 160, 167, 173	275, 277, 279, 281, 285, 288, 292, 296, 310	257, 269, 272, 275, 278, 281	111, 132, 136, 138	241, 244	263, 267	240, 243	33:3
DD	202, 205, 208, 211, 214	153, 157, 161, 164, 165, 166, 171, 173, 175, 176, 177, 192, 194	181, 186, 189, 203, 204, 206, 210, 211, 213, 216, 217, 222, 230	155, 160, 162, 173	275, 277, 279, 281, 283, 285, 287, 288, 292, 294, 296, 298, 308	266, 269, 272, 275, 278, 281	105, 111, 113, 132, 136, 138	241, 244	263, 264, 265, 267, 269	240, 243	29:2
BH	197, 202, 205, 208, 211, 214, 220	153, 157, 161, 165, 166, 171, 173, 175, 176, 179	181, 189, 195, 203, 204, 211, 216, 217	155, 160, 167, 173	275, 277, 279, 281, 283, 285, 287, 288, 290, 294, 296, 298, 300, 306, 308, 314	266, 269, 273, 275, 278, 288	111, 121, 130, 132, 136, 138	240, 241, 244, 248	263, 265, 267, 269	240, 243	34:2
KH	205, 208, 211	153, 157, 161, 165, 166, 171, 173, 174, 175, 176, 192, 198	181, 183, 189, 195, 203, 204, 208, 211, 216, 217, 222	155, 160, 167, 173, 179	275, 277, 279, 281, 283, 285, 287, 288, 290, 292, 294, 296, 304, 306, 314	266, 269, 273, 275, 276, 288	111, 132, 138, 163	236, 241, 244, 248	263, 265, 267, 269	240, 243	31:3
RC	205, 208, 211, 220	153, 157, 161, 165, 166, 171, 173, 175, 179	181, 189, 190, 195, 203, 204, 216, 211	155, 160 167, 173, 179	275, 277, 279, 281, 283, 285, 287, 288, 290, 296, 306, 314	269, 273, 275, 278, 288	111, 132, 138	240, 241, 244, 248	263, 265, 267, 269	240, 243	13:0
TH	202, 205, 208, 211	151, 153, 157, 161, 164, 165, 166, 171, 173, 175, 176, 192, 194, 211	181, 182, 183, 186, 189, 203, 204, 206, 211, 216, 217, 222, 230,	155, 160, 167, 173	273, 275, 277, 279, 281, 283, 285, 287 288, 290, 292, 294, 296, 298, 306	266, 269, 271, 272, 273, 275, 278, 281, 288, 294	111, 132, 136, 138, 139, 161	236, 240, 241, 244	262, 263, 265, 267, 268, 269	240, 243, 246	31:8
AU	205, 208, 211	153, 161, 166, 173, 176	189, 203, 204, 211	160, 167, 173	275, 277, 285, 288, 290, 294	269, 275	132, 138	242, 244	263, 265	240, 243	8:0
SH	205, 211	161, 165, 166, 171, 175	181, 189, 204, 211	155, 160, 167, 173	277, 279, 281, 283, 285, 296, 304, 306	269, 275, 278	111, 136, 138, 132	244, 248	263, 265	240, 243	12:0
MD	205, 211, 233, 235	161, 165, 173, 175, 176, 179	181, 183, 195, 204, 216, 222	155, 160, 167, 173	277, 279, 281, 285, 287, 288, 306	269, 272, 278	111, 128, 130, 132, 134, 136, 138, 141	244, 236	263, 265	233, 240, 243	9:4
BW	197, 205, 208, 211, 226	161, 164, 165, 166, 171, 173, 175, 176	181, 183, 189, 204, 211, 213, 216, 222, 225	155, 160, 167, 173	275, 277, 279, 283, 285, 287, 288, 290	266, 269, 272, 275	111, 132, 136, 138	241, 242, 244, 248	263, 265, 269	240, 243	25:0
HG	197, 205, 211, 220	161, 165, 166, 171, 175, 176	181, 183, 204, 216, 222	155, 160, 167, 173	275, 277, 279, 285, 287, 288, 290, 296	269, 275	111, 132, 136	241, 244, 248	263, 265	240, 243	15:0
BD	197, 200, 205, 208, 211, 226	153, 161, 166, 176, 171, 173, 175	181, 183, 189, 197, 199, 204, 210, 211, 216	155, 160, 167, 173	275, 277, 279, 285, 287, 288, 281, 290, 296	266, 269	111, 132, 136, 138	241, 244	263, 269	240, 243	30:0
HR	197, 200, 205, 208, 211	161, 164, 165, 173, 175, 176	181, 183, 189, 201, 197, 204, 236, 237	155, 160, 167, 173	273, 275, 277, 285, 287, 288, 294, 314	266, 269, 275	111, 132, 138	241, 244	263, 269	239, 240, 242, 243	34:5

Appendix 5: Raw microsatellite data for regenerated/restored populations of *Anemone pulsatilla* L. and wild provenance populations

- Microsatellite alleles recorded for each population under 10 loci
- Microsatellite alleles listed in base pair length order
- Unique population alleles (of wild provenance population) in red text
- Wild provenance population highlighted in green
- Restored/regenerated population highlighted in yellow

BH	197, 202, 205, 208, 211, 214, 220	153, 157, 161, 165, 166, 171, 173, 175, 176, 179	181, 189, 195, 203, 204, 211, 216, 217	155, 160, 167, 173	275, 277, 279, 281, 283, 285, 287, 288, 290, 294, 296, 298, 300, 306, 308, 314	266, 269, 273, 275, 278, 288	111, 121, 130, 132, 136, 138	240, 241, 244, 248	263, 265, 267, 269	240, 243
HA	197, 200, 202, 205, 208, 211, 220	153, 157, 161, 165, 166, 171, 173, 175, 192	181, 182, 189, 201, 202, 203, 204, 211, 216, 222	155, 160, 167, 173, 179	273, 275, 277, 279, 281, 283, 285, 287, 288, 290, 292, 294, 296, 308, 314,	263, 266, 269, 272, 273, 275, 278, 288	111, 132, 136, 138	241, 244, 248	263, 265, 267	240, 243, 246
BA	202, 205, 208, 211	153, 157, 161, 165, 166, 173, 174, 175, 176, 177, 194	181, 182, 189, 204, 211, 213, 216, 221, 222, 225, 227, 229	155, 160, 167, 173	275, 277, 279, 281, 285, 288, 292, 296, 310	257, 269, 272, 275, 278, 281	111, 132, 136, 138	241, 244	263, 267	240, 243
JL(BA) 2013	197, 200, 202, 205, 211	153, 157, 166, 173, 174,	181, 189, 204, 216, 222, 227	155, 160, 167	275, 277, 279, 281, 285, 287, 288, 296, 314,	257, 269, 275, 278, 281	111,132, 136,	241, 244	263, 267	240, 243
AR	197, 200, 202, 205, 211	153, 157, 161, 165, 166, 171, 173, 174, 175, 176	181, 182, 189, 204, 211, 213, 216, 221, 227	155, 150, 167, 173	275, 277, 279, 281, 283, 285, 292, 296, 306	257, 269, 272, 275, 278	111, 132, 136, 138	241, 244, 248	263, 265, 267	240, 243
WP(BA) 2011	197, 200, 202, 205, 211	153, 157, 161, 165, 166, 173, 174, 175, 176, 179	181, 182, 189, 204, 211, 216, 221, 227	155, 160, 167, 173	275, 277, 279, 281, 285, 296, 314	257, 269, 272, 275, 278,	111, 113, 132, 136, 139	241, 244, 248	263, 265, 267	240, 243
WP(BA) 2012	200, 202, 205, 208, 211	153, 161, 165, 166, 173, 174, 175, 179	181, 182, 189, 203, 204, 216, 221, 227	155, 160, 167	275, 277, 281, 285, 296	257, 269, 275, 278, 281	111, 132, 136, 138	241, 244	263, 265, 267, 269	240, 243
WP(BA) 2013	197, 200, 202, 205, 208, 211	153, 157, 161, 165, 166, 173, 174, 175, 176	181, 182, 189, 204, 211, 216, 221, 225	155, 160, 167	275, 277, 281, 285, 288, 296, 310	257, 269, 272, 275, 278	111, 132, 136	241, 244	263, 265	240, 243
WP(BA) 2014	197, 200, 202, 205, 211,	153, 157, 161, 165, 166, 173, 174, 175, 202	181, 182, 189, 203, 204, 211, 221, 216, 227,	155, 160, 167, 173	275, 277, 279, 281, 285, 287, 288, 292, 296, 314	257, 269, 275, 278, 281	111, 132, 136	241, 244	263, 265, 267	240, 243
WP(BA) 2012_ F2	197, 200, 202, 205, 211	153, 157, 161, 165, 166, 173, 174, 175	181, 182, 189, 204, 216, 221	155, 160, 167	275, 277, 279, 281, 285, 292, 296	257, 269, 275, 278	111, 132, 136	241, 242, 244	263, 267	240, 243
SH	205, 211	161, 165, 166, 171, 175	181, 189, 204, 211	155, 160, 167, 173	277, 279, 281, 283, 285, 296, 304, 306	269, 275, 278	111, 136, 138, 132	244, 248	263, 265	240, 243
JL(SH) 2013	197, 200, 205, 208, 211	161, 165, 166, 171, 175	181, 189, 204, 211,	155, 160, 167, 173	275, 279, 283, 285, 296, 306	269, 275, 278	111,, 136, 138	244, 248	263, 265	240, 243
AU	205, 208, 211	153, 161, 166, 173, 176	189, 203, 204, 211	160, 167, 173	275, 277, 285, 288, 290, 294	269, 275	132, 138	242, 244	263, 265	240, 243
AU_AU	197, 200, 205, 208, 211	153, 166, 173	181, 189, 203, 204, 216	160, 167, 173	275, 277, 285, 288,, 290, 294	269, 275	132, 136, 138	242, 244	263, 265	240, 243

Appendix 6: Raw microsatellite data for casually restored population Martin Down

- STRUCTURE input file
- Unique population alleles in red

	JSZZ3	JVIKZ	JQ389	41A30S	JZD0G	gr105_4	IAJN8	IHA0O	ID331	I6F9M
MD134	205	161	181	155	277	269	111	244	263	243
MD134	211	165	183	160	285	278	0	0	265	0
MD134	0	173	204	173	287	0	0	0	0	0
MD134	0	175	216	0	0	0	0	0	0	0
MD135	205	165	183	155	277	269	111	244	265	243
MD135	211	173	204	160	285	278	0	0	0	0
MD135	0	175	216	173	0	0	0	0	0	0
MD135	0	0	0	0	0	0	0	0	0	0
MD136	205	161	181	155	277	269	132	244	263	240
MD136	0	165	222	160	279	278	0	0	0	243
MD136	0	176	0	167	285	0	0	0	0	0
MD136	0	0	0	0	287	0	0	0	0	0
MD137	205	165	181	155	277	269	111	244	263	240
MD137	0	176	222	173	287	278	136	0	0	243
MD137	0	0	0	0	0	0	0	0	0	0
MD137	0	0	0	0	0	0	0	0	0	0
MD138	211	161	181	160	281	272	**128**	236	265	**233**
MD138	**233**	179	195	167	288	0	141	0	0	240
MD138	**235**	0	0	0	0	0	0	0	0	0
MD138	0	0	0	0	0	0	0	0	0	0
MD139	205	161	181	155	277	269	111	244	263	240
MD139	0	175	204	160	285	278	130	0	265	243
MD139	0	0	222	167	287	0	134	0	0	0
MD139	0	0	0	173	306	0	138	0	0	0
MD140	205	161	181	155	277	269	111	244	263	240
MD140	0	175	204	160	285	278	136	0	265	243
MD140	0	0	222	167	287	0	0	0	0	0
MD140	0	0	0	173	306	0	0	0	0	0
MD141	205	161	181	155	277	269	136	244	263	240
MD141	0	175	204	160	287	278	0	0	265	243
MD141	0	0	222	167	306	0	0	0	0	0
MD141	0	0	0	173	0	0	0	0	0	0
MD142	205	161	181	155	277	278	111	244	263	240
MD142	0	175	204	160	287	0	136	0	265	243
MD142	0	0	0	167	306	0	0	0	0	0
MD142	0	0	0	173	0	0	0	0	0	0

Appendix 7: Raw microsatellite data for casually restored population Ancaster Valley

- STRUCTURE input file
- Unique population alleles in red

	JSZZ3	JVIKZ	JQ389	41A30S	JZD0G	gr105_4	IAJN8	IHA0O	ID331	I6F9M
AN327	202	161	189	155	275	260	134	240	263	240
AN327	0	175	216	167	281	0	0	0	0	0
AN327	0	0	0	173	0	0	0	0	0	0
AN327	0	0	0	0	0	0	0	0	0	0
AN328	197	161	189	155	275	260	134	240	263	240
AN328	202	175	216	160	287	0	141	0	0	0
AN328	208	194	0	167	308	0	0	0	0	0
AN328	0	0	0	173	0	0	0	0	0	0
AN329	208	161	189	155	275	260	134	240	263	240
AN329	0	175	216	160	281	0	141	0	0	246
AN329	0	194	0	167	287	0	0	0	0	0
AN329	0	0	0	173	0	0	0	0	0	0
AN330	197	161	189	155	275	260	134	240	263	240
AN330	202	194	216	167	281	0	141	0	0	246
AN330	208	0	0	173	287	0	0	0	0	0
AN330	0	0	0	0	308	0	0	0	0	0
AN331	202	161	189	160	281	260	132	244	263	240
AN331	205	174	230	167	288	275	136	0	265	243
AN331	0	176	0	0	308	0	0	0	0	0
AN331	0	0	0	0	0	0	0	0	0	0
AN332	208	161	189	155	275	260	141	240	263	240
AN332	0	175	216	160	287	0	0	0	0	246
AN332	0	194	0	167	0	0	0	0	0	0
AN332	0	0	0	173	0	0	0	0	0	0
AN333	197	161	221	155	275	275	138	240	265	243
AN333	205	176	230	160	287	0	0	244	267	246
AN333	208	0	0	167	288	0	0	0	0	0
AN333	0	0	0	0	296	0	0	0	0	0
AN334	205	153	216	160	281	275	130	244	263	243
AN334	208	161	**219**	167	287	0	141	0	265	0
AN334	0	166	0	173	292	0	0	0	0	0
AN334	0	**181**	0	0	296	0	0	0	0	0
AN335	202	161	189	155	281	260	134	240	263	246
AN335	0	175	216	160	287	0	141	0	0	0
AN335	0	0	0	167	308	0	0	0	0	0
AN335	0	0	0	173	0	0	0	0	0	0
AN336	202	161	189	155	275	260	134	240	263	240
AN336	208	175	216	160	281	0	141	0	0	246
AN336	0	194	0	167	287	0	0	0	0	0
AN336	0	0	0	173	308	0	0	0	0	0

	JSZZ3	JVIKZ	JQ389	41A30S	JZD0G	gr105	4 IAJN8	IHA0O	ID331	I6F9M
AN337	202	161	216	160	275	260	134	240	263	246
AN337	0	194	0	167	281	0	141	0	0	0
AN337	0	0	0	0	287	0	0	0	0	0
AN337	0	0	0	0	308	0	0	0	0	0
AN338	197	161	189	160	275	260	134	240	263	240
AN338	208	175	216	167	281	0	141	0	0	0
AN338	0	194	0	0	287	0	0	0	0	0
AN338	0	0	0	0	0	0	0	0	0	0
AN339	197	161	189	155	281	260	134	240	263	246
AN339	202	175	216	160	287	0	0	0	0	0
AN339	0	194	0	173	308	0	0	0	0	0
AN339	0	0	0	0	0	0	0	0	0	0
AN340	197	161	189	155	281	260	134	240	263	240
AN340	202	175	216	167	287	0	141	0	0	246
AN340	0	194	0	0	308	0	0	0	0	0
AN340	0	0	0	0	0	0	0	0	0	0
AN341	208	161	189	160	275	260	111	240	263	240
AN341	0	175	216	167	281	0	134	0	0	246
AN341	0	0	0	173	0	0	141	0	0	0
AN341	0	0	0	0	0	0	0	0	0	0
AN342	197	161	189	160	275	260	134	240	263	246
AN342	202	175	216	167	281	0	0	0	0	0
AN342	0	194	0	173	287	0	0	0	0	0
AN342	0	0	0	0	308	0	0	0	0	0
AN343	**202**	161	204	155	277	257	132	244	263	243
AN343	208	174	0	160	279	281	134	252	0	0
AN343	0	202	0	167	290	0	0	0	0	0
AN343	0	0	0	0	296	0	0	0	0	0
AN344	202	161	189	155	275	260	141	240	263	240
AN344	208	175	216	167	287	0	0	0	0	246
AN344	0	0	0	0	308	0	0	0	0	0
AN344	0	0	0	0	0	0	0	0	0	0
AN345	197	161	181	160	287	260	136	240	265	243
AN345	202	166	**219**	167	296	275	0	244	0	246
AN345	**231**	0	222	0	308	0	0	0	0	0
AN345	0	0	0	0	0	0	0	0	0	0
AN346	197	161	204	155	277	257	132	244	263	243
AN346	202	174	0	160	279	281	134	252	0	0
AN346	208	202	0	167	290	0	0	0	0	0
AN346	0	0	0	0	296	0	0	0	0	0