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Conservation genetics of montane willow populations in Scotland—limited natural recovery despite long-distance gene flow and high genetic diversity

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Abstract

Plant species around the world are negatively affected by habitat fragmentation and climate change. Montane willow populations in the UK have declined primarily due to grazing, as well as climate change and muirburn in certain areas. Only small, isolated populations remain, scattered across steep ledges where shrubs escape most grazing animals. We explored the genetic effects of habitat fragmentation on gene flow and genetic diversity in the largest remaining patches in the UK, which lie in Scotland, for two now restricted and rare montane willow species—*Salix myrsinites* and *Salix lapponum*. Using ten microsatellite loci and an almost complete genetic inventory in the central Cairngorms area (*S. myrsinites* $n = 186$, *S. lapponum* $n = 91$) we showed that genetic diversity (heterozygosity and allelic richness) is relatively high in both species, particularly high for the decaploid *S. myrsinites*, and clonal reproduction low. Historic gene flow between populations could be demonstrated. Significant inbreeding was detected in both species and observed seed set and numbers of juveniles in the field are low, possibly indicating signs of inbreeding depression. Both species have extremely low numbers of individuals at each site, with many being smaller than ten, and in some cases combined with skewed sex-ratios (mainly female biased). This will likely cause more severe reproductive failures in the next generations unless population numbers are increased. Reinforcing populations of both species under low grazing pressure with new, unrelated individuals, as well as creating new stepping-stone populations will be necessary to help the recovery of these species. Results from this study will inform restoration work in Scotland aiming to create continuous woodland habitats from pinewoods at lower altitudes through to higher altitude downy birch woodlands merging with montane willows.

1. Introduction

Habitat fragmentation, habitat loss and climate change pose the most severe current and future threats to plant biodiversity globally and around two in five plant species are already threatened with extinction (Lughadha *et al* 2020). Many of these threatened species are only left in fragments which are small, isolated and in poor reproductive condition. This is not only making them prone to extinction due to random events (e.g. grazing, landslides or disease) but also due to negative genetic effects such as the loss of genetic diversity or inbreeding depression (Spielman *et al* 2004, Frankham 2005). Increasing numbers of individuals in populations, reducing distances between populations to allow gene flow and keeping relatedness low is a

priority for conservation and restoration. Mixing unrelated individuals from different populations, i.e. genetic rescue, has been a successful tool for reversing negative impacts of inbreeding depression and maximising evolutionary resilience for plant species (Finger *et al* 2011, Weeks *et al* 2011, Oakley *et al* 2015, Whiteley *et al* 2015, Zavodna *et al* 2015, Van Rossum and Hardy 2021).

There is the potential though to introduce maladapted genotypes which may lower reproductive fitness of hybrid offspring (outbreeding depression) due to the interruption of local co-adapted gene complexes (Tallmon *et al* 2004, Edmands 2007, Barmentlo *et al* 2018). Outbreeding depression is predictable though, and risks are low if mixing populations from similar habitats or those that have been connected in the past (Frankham *et al* 2011, 2017). To determine best suited conservation intervention for rare species and minimise potential risks from inbreeding or outbreeding depression it is therefore important to use genetic data on species prior to on-the-ground conservation efforts.

Montane willow populations, a component of montane woodlands, are very rare and severely fragmented in Scotland due primarily to a history of grazing and burning (Oosthoek 2013). It is possible that following long-term population isolation, montane willows are struggling to regenerate naturally due to genetic problems which would make their restoration more challenging (Charlesworth and Charlesworth 1999, Leimu *et al* 2010, Angeloni *et al* 2011, Hedrick and Garcia-Dorado 2016). Climate change will likely further affect their persistence in the landscape due to their northerly distribution and occurrence at high altitudes (IPBES 2019). These species may be forced to retreat from certain parts of their present distribution should climatic warming continue. Reduced winter snow cover, particularly early snow melt could leave plants susceptible to browsing, increased fungal damage, reduced fruit set, and a reduced genetic diversity, as already documented for other willow species (Cortés *et al* 2014, Wheeler *et al* 2016). To maximise the chances of their survival as well as their ability to regenerate and expand in a changing climate, existing populations are in urgent need of restoration.

The two largest remaining patches of montane willows in the UK are in Scotland, in the Northern Cairngorms. Considerable work is being undertaken in this area for landscape scale restoration and expansion of montane woodlands, including montane willow populations (e.g. Cairngorms Connect, Mar Lodge). The two rare and threatened montane willow species *Salix myrsinites* and *Salix lapponum* only remain as small and fragmented populations but nothing is known about the partitioning of their genetic diversity in this area. Not only are populations small but they are also dioicous (Rechinger 1992, Ming *et al* 2011) which could create skewed sex ratio in populations, further reducing the likelihood of successful pollination. Furthermore, willow species are known to hybridise, particularly when one species is significantly outnumbered by another, adding a taxonomic challenge.

To prevent negative genetic effects from small populations and minimise the risk of outbreeding depression during restoration work we explored the genetic diversity and connectivity of small and fragmented montane willow populations, *S. myrsinites* and *S. lapponum*. This information will help to determine best suited source populations for conservation translocations. To this end we have conducted a complete population survey and genetic inventory across three estates in Scotland using polymorphic microsatellite loci to assess (a) population sizes, sex ratios and rates of reproduction (b) the amount of remaining genetic diversity, (c) the amount of genetic connectivity, (d) and explored the possibility of identifying hybrids. The results from this study will help inform applied conservation work on the ground but also the management and restoration of other rare species.

2. Material and methods

2.1. Study species

In the UK both *S. myrsinites* and *S. lapponum* are low spreading shrubs growing primarily on moist or wet base-enriched sites on rocky mountain slopes, gulleys or cliffs. *S. lapponum* tolerates a wider range of soil conditions than most montane willow species. Both species have a wide distribution in north and north-eastern Europe. *S. lapponum* distribution extends eastwards to western Siberia and as far south as the Pyrenees and Bulgaria. *S. myrsinites* is not found in the mountains of central Europe. In Scotland both species have similar altitudinal ranges from approximately 200 m (in the Ochils) to 1000 m. However, in the inland Cairngorm Mountains both species are usually found between 600 and 1050 m (Beck 2019 unpublished report for NTS, available on request). Both *S. lapponum* and *S. myrsinites* are dioecious, developing male and female flowers in different individuals. The species are insect pollinated and the seeds wind dispersed. *S. lapponum* tends to hybridise more readily than *S. myrsinites*. There are 68 reported hybrids in the *Salix* genus (Stace 2019), sometimes between three different species and this extensive degree of hybridisation in addition to morphological variations within species (Rechinger 1992) makes willow identification very challenging. While it is not known how many *S. lapponum* or *S. myrsinites* hybrids are

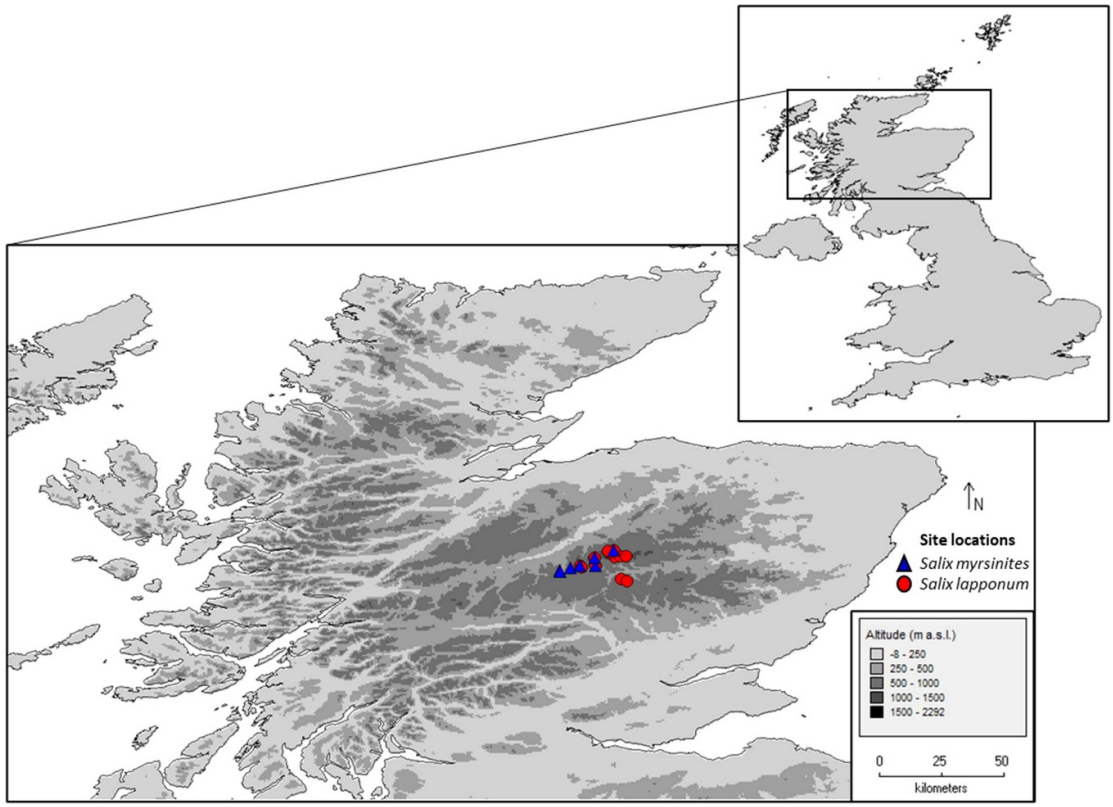

Figure 1. Overview of *Salix myrsinites* (blue triangles) and *S. lapponum* (red circles) sample locations in the Cairngorms National Park, Scotland.

present in the study area, the only other willow species occurring within pollination distance are *Salix aurita, Salix herbacea, Salix phyllicifolia, Salix myrsinifolia, Salix caprea* and *Salix cinerea*. However, all of these, possibly with the exceptions of *S. herbacea* are very rare in the uplands.

They are palatable to herbivores and in areas where wild or domestic herbivores are at high densities grazing can impact significantly on the populations. In Scotland centuries of heavy grazing by sheep and deer, and historically goats, have resulted in the decline and range contraction of these species. In certain areas such as the Cairngorms National Park conservation efforts have resulted in a significant reduction in grazing pressure with a view to restoring and expanding montane willows and other woodland types and these areas would be suitable for e.g. population augmentation or conservation translocations. A large landscape scale restoration project is currently underway which includes the restoration of montane willow habitat. This is a partnership of neighbouring land managers to enhance habitats, species and ecological processes across the Cairngorms National Park (http://cairngormsconnect.org.uk/; https://rewildingbritain.org.uk/rewilding-projects/mar-lodge).

2.2. Sampling

All existing populations of both species in the study area, across three Estates (Glenfeshie, Mar Lodge and Abernethy) in the Cairngorms National Park, have been monitored to determine the remaining number of individuals, browsing damage, and male/female ratios in populations.

A complete genetic inventory has been undertaken in the study area in 2019. In total 190 *S. myrsinites* leaf samples from individual plants were analysed from seven locations. After removing all clones and samples that were not *S. myrsinites*, 186 samples remained for analysis. One hundred and eleven *S. lapponum* leaf samples were collected from ten locations. After removing all clones, potential hybrids and samples of low DNA quality the following analyses were based on 91 samples.

For the purpose of this study populations were defined according to their geographic locations as well as the STRUCTURE results (see details below). For *S. lapponum* this resulted in four populations/genetic clusters, SL1-4, combining all individuals from the south and west into one population. We also analysed the same dataset based on eight populations and low pairwise differentiation between populations (data not shown) confirms that using four populations is appropriate. For *S. myrsinites* using information from STRUCTURE and the geographic locations resulted in five populations, SM1-SM5. An overview of population locations is given in figures 1 and 3.

All calculations will be based on these population definitions, though calculations based on four populations for *S. lapponum* may ignore some sub-structuring in populations which could cause artificially increased inbreeding values (Wahlhund effect) and this was taken into account when analysing the data.

2.3. Microsatellite marker development and genetic analyses

Enriched libraries were created for *S. myrsinites* by Ecogenics (www.ecogenics.ch/home.html). Primer development took place as described by Ecogenics: 'The Illumina TruSeq nano library was analysed on an Illumina MiSeq sequencing platform using a nano v2 500 cycles sequencing chip. The resulting paired-end reads which passed Illumina's chastity filter were subject to de-multiplexing and trimming of Illumina adaptor residuals. Subsequently the quality of the surviving reads was checked with the software FastQC v0.117. In a next step the paired-end reads were merged with the software USEARCH v10.0.240 to *in-silico* re-form the sequenced molecule. The resulting merged reads were screened with the software Tandem Repeats Finder, v4.09. After this process, 14 462 merged reads contained a microsatellite insert with a tetra- or a trinucleotide of at least 6 repeat units or a dinucleotide of at least 10 repeat units. Primer design was performed with primer 3. Suitable primer design was possible in 7937 microsatellite candidates'.

2.4. DNA extraction and polymerase chain reaction (PCR) conditions

Of the 7937 microsatellite candidates, only the ten most variable loci were optimised for *S. myrsinites* (1, 3, 4, 6, 7, 9, 12, 19, 20, 21) and labelled with an M13-tag at its 5′-end described by Schuelke (2000). For *S. lapponum* eleven markers were used, five markers cross-amplified well from those developed for *S. myrsinites* (1, 4, 11, 12, 20) and a further six (CT11, 446, CT52, 223, 475, CT24) were used from other studies (Stamati *et al* 2003, Lexer *et al* 2005, Hoshikawa *et al* 2009, Puschenreiter *et al* 2010), see table 1. For *S. myrsinites* all markers showed significant deviations from Hardy Weinberg Equilibrium (HWE) (data not shown) and it is therefore likely that deviations are due to inbreeding in populations rather than null alleles. For *S. lapponum* SSR 223 was the only marker with significant deviations from HWE and had more missing data compared to the other markers, hinting towards null alleles, and was therefore excluded from the analysis.

Genomic DNA was extracted from silica dried leaves using the QIAGEN DNeasy 96 Plant Kit, following the manufacturer's protocol. PCR was carried out in 10 μl reactions with 1 μl of 1X PCR buffer (Promega colorless Flexi GoTaq PCR buffer), 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.05 μl of the 0.1 μM M13 forward primer, 0.1 μl of the 0.1 μM reverse primer and 0.1 μl of the 0.1 μM M13 primer, 0.03 U Taq polymerase (Promega), and 2 μl DNA template (c. 10 ng). Cycling conditions were as follows: 1 $\times$ (94 °C for 5 min), 30 $\times$ (94 °C for 45 s, primer-specific temperature (57 °C) for 30 s, 72 °C for 30 s), 1 $\times$ (72 °C for 30 min) carried out in a Bio-Rad Dyad Cycler. Fragment analysis was conducted using an ABI3730 sequencer (University of Edinburgh) and genotyped using the program Genemarker v.2.7.0.

2.5. Data analyses

S. myrsinites is decaploid and population genetic analysis are limited due to the complexity of the analysis of such a dataset. Analytical limitations include problems to determine allele copy numbers in partial heterozygotes, inferring allele frequencies and assumptions regarding inheritance (Bruvo *et al* 2004, De Silva *et al* 2005, Obbard *et al* 2006, Dufresne *et al* 2014, Meirmans *et al* 2018). An overview of complications arising during analyses of polyploid data and approaches to minimise statistical bias is provided in Meirmans *et al* (2018). We followed their recommendations on statistical analyses and data interpretation. Some analyses were done using the R package Polysat (Clark and Jasieniuk 2011), a software specifically developed to work with microsatellite data of any ploidy level. It uses an iterative allele frequency estimation to analyse data with allele copy number ambiguity and is currently the most accurate allele frequency estimation implemented for autopolyploids (Clark and Jasieniuk 2011). Polysat also creates a co-dominant matrix for further analysis in other programs that allow for polyploid datasets, such as STRUCTURE v.2.3.4 (Pritchard *et al* 2000) and SPAGeDi v.1.5 (Hardy and Vekemans 2002), which can deal with polyploid datasets. We have also created a co-dominant matrix by estimating allele copy numbers of partial heterozygotes by assigning number of allele copies proportionate to their peak height in Genemapper (Pfeiffer *et al* 2011). We found that this produced more robust results compared to the data matrix produced by Polysat and therefore used this for further analysis in SPAGeDi and STRUCTURE. *S. lapponum* is diploid and data analysis was therefore straightforward.

2.5.1. Assessment of genetic diversity, clonal reproduction, inbreeding and kinship

For both species number of alleles (N_A), the number of effective alleles (N_E), observed heterozygosity (H_O), gene diversity corrected for sample size, also referred to as expected heterozygosity (H_E), and allelic richness (A_R), number of alleles corrected by rarefaction, were calculated in SPAGeDI. Global inbreeding

Table 1. Characteristics of polymorphic microsatellite loci in *Salix lapponum* and *S. myrsinites*.

Species	Locus	Genbank accession	Primer sequence (5′-3′)	Repeat motif	Size range (bp)	T_a (°C)	N_A
SM + SL	*1*	ON676485	F AGTCACGGGCCAATTAAAGG R CACACGTATGCCACCTTCTC	$(TTA)_9$	107–213	57	38
SM	*3*	ON676486	F ACTGGTATGCAG-GAATTTTAGTCTG R TACCTGATGCAGGACACCG	$(TAA)_8$	118–336	57	33
SM + SL	*4*	ON676487	F TCATAGCCTTCGAGTCGTCC R ACATTTTGCCCCAGTTAGCG	$(TCC)_{16}$	151–233	57	25
SM	*6*	ON676488	F TCTTGATGGAAATAAAATT CAAACGC R CTATTCGAGGGGCAAAACCG	$(ATA)_8$	256–327	57	26
SM	*7*	ON676489	F TACGGTGGCATTGTTTTCCC R AGATAACACGACGACCTGCG	$(AAT)_9$	113–279	57	26
SM	*9*	ON676490	F GGACTTCTAA CTTTTTGCAGCG R GCTCCATACCTGCGGAAATC	$(TTC)_{10}$	181–366	57	52
SL	*11*	ON676491	F CGGTACATTCAGATTTGACT-TGATG R TCCATACCTTTTCGGGCAGG	$(TAA)_8$	221–263	57	13
SM + SL	*12*	ON676492	F GCATGTTACCATCTT TCTTTCGC R GCACAGCTG-ATCTAAATTCAAAACC	$(AAG)_8$	146–216	57	22
SM	*19*	ON676493	F CCCCAGTTACTTCCGAGTTG R AGGTACAACCAGCCTCGAC	$(TTTA)_7$	208–274	57	16
SM + SL	*20*	ON676494	F ACTCCAAAATGGGAATGTGCC R ATGGAGGTCCCACAACCTTC	$(ATGT)_7$	93–181	57	40
SM	*21*	ON676495	F ACGTTGCTTCTTAGTGATGCG R GTCGCCTTGACAGTTCTCAC	$(AAT)_{12}$	94–199	57	37
SL	*CT11*	—	F ACTCCAAAATGGGAATGTGCC R ATGGAGGTCCCACAACCTTC	$(CT)_{11}$	303–335	57	6
SL	*446*	—	F CGGGCTGCAGACAAATTAAGG R TGGGACATGCTCCATGGTAT	$(CT)_{3...}(CT)_4$	226–268	57	11
SL	*CT52*	—	F TTCTTTTTCCACTCGCCAC R GGATTGACCCATCTCGATTC	$(CT)_{15}(AG)_{20}$	255–341	57	19
SL	*223*	—	F CCGATGAGGTTGAAGAAGTCG R ATATATGTACCGGCACGCCAC	$(CTT)_n$	183–204	57	3
SL	*475*	—	F CAGGGAATGAGAGATGGTA-GAGT R GGGAAGGTAAGTTGGTGTTG	$(GT)_{12}(GA)$	154–172	57	8
SL	*CT24*	—	F CTCATTTGCTCGATGAGGTTG R GTGGTAGTTGCAAAAGGGGA	$(CT)_{10}$	295–345	57	14

Abbreviations: SM: *Salix myrsinites*; SL: *Salix lapponum*; F: forward primer; R: reverse primer; T_a: annealing temperature; N_A: number of alleles; H_o: observed heterozygosity; H_e: expected heterozygosity; 190 *S. myrsinites* individuals were analysed for each locus and for *S. lapponum* 111 individuals were analysed. Loci CT11, CT52 and CT24 from Stamati *et al* (2003), locus 446 from Puschenreiter *et al* (2010), locus 223 from Lexer *et al* (2005), locus 475 from Hoshikawa *et al* (2009).

coefficients (F_{IS}), computed for all loci and population specific inbreeding coefficient F_i (using 1000 permutations) were calculated as a multilocus average using SPAGeDI.

All samples for both species were compared at ten microsatellite loci. Samples that showed the same allele combination across all loci (allowing a maximum of three missing data per individual) were assumed to be the same individual.

Multilocus kinship coefficients were calculated for all possible pairwise combinations for all genotypes and populations and mean kinship coefficients for each population estimated using SPAGeDi. The kinship coefficient is computed as a correlation coefficient between allelic states proposed by J Nason (Loiselle *et al* 1995, SPAGeDi manual). Due to the complexity around analysing polyploid data, we only calculated kinship values for *S. lapponum*.

2.5.2. Assessment of genetic structure over the species range

To determine the genetic structure between populations of *S. myrsinites* a pairwise distance matrix between all populations was calculated (using Bruvo distances between individuals) and a principal coordinate

analysis (PCoA) performed in Polysat. Polysat calculates Bruvo distance by counting each allele as being present in a copy, similar to band-sharing measures but takes mutational distance between microsatellite alleles into account and is therefore suitable for accounting for a stepwise mutation model (Bruvo *et al* 2004, Clark and Jasieniuk 2011). For *S. lapponum* a PCoA was performed in GenAlEx (Peakall and Smouse 2006).

We also tested for the presence of geographical groupings of related samples by applying a Bayesian cluster analysis to all individuals using the software STRUCTURE. For *S. myrsinites* we carried out a total of 150 runs, ten each for one to fifteen clusters (K1 to K15) and for *S. lapponum* 200 runs, ten each for one to fifteen clusters (K1 to K20). For each run the burn-in and simulation length was 30 000 and 50 000, respectively. To identify the most likely number of distinct genetic groups (K) in the dataset we used two different calculations. The ΔK statistic, which is based on the rate of change in the log probability of data between successive K values (Evanno *et al* 2005), as well as $\Pr[X|K]$ (the probability of obtaining the genotype data X given K) of Pritchard *et al* (2000) were calculated using the online version of the software CLUMPAK (Kopelman *et al* 2015), http://clumpak.tau.ac.il.

For populations with a high H_S values, which is often the case for highly variable markers such as microsatellites, F_{ST} values will be low, even when gene flow is absent among populations ($F_{ST}(\max) = 1 - H_S$) (Meirmans and Hedrick 2011, Meirmans *et al* 2018). The most relevant F_{ST} alternative for autotetraploids are the Rho (ρ) and Jost's D statistics (2008). Rho is convenient to compare the level of genetic structuring among ploidy levels but values may be underestimated with highly polymorphic markers. Jost's D is useful in such cases, since its value is also ploidy independent as well as independent of population size and uses the effective number of alleles to quantify diversity (Meirmans *et al* 2018). For the analyses in this study, we only present Jost's D values as they were very similar to the Rho values, both have been calculated in Polysat for *S. myrsinites* and Jost's D values calculated in GenAlEx for *S. lapponum*.

2.5.3. Assessment of hybridisation

To test whether some sampled shrubs were hybrids rather than pure species we tried to establish species-specific alleles from known pure species which are not present in known hybrids. Furthermore, we used ploidy in *S. lapponum* to identify some hybrids. Hybridisation was assumed when markers showed more than two alleles at a given loci and/or despite high quality DNA a lot of markers did not amplify. If more than two alleles were detected in an individual at several markers it was assumed that it is not a mistake during PCR but rather polyploidy arisen from hybridisation. Furthermore, we use morphological field observations in combination with generic data to distinguish between hybrids.

3. Results

3.1. Population surveys

The three estates were thoroughly surveyed in 2019 to identify every site containing either study species and sites were subsequently monitored. Very little recruitment was found despite grazing pressure being reduced across most site since the early 2000s. For *S. myrsinites* nine juveniles were found in total within two (out of seven) sites. For *S. lapponum* four juveniles were found in total, one in each of four (out of twelve) sites, see table 2.

Eight out of twelve *S. lapponum* sites consisted of less than ten individuals and most sites had more female than male plants, at six sites only female plants were found. Gender was more balanced for *S. myrsinites* sites though all populations also have an excess of female plants. The gender of some plants for both species could not be identified as they were not flowering, and it is possible that some of these figures would change slightly if gender was known. Browsing damage was apparent at most sites for both species, with an average browsing damage per plant below 20% for most sites and a maximum of 33.13% (ML9, *S. lapponum*).

3.2. Genetic diversity, clonal growth, inbreeding and kinship

3.2.1. S. myrsinites

At the species level (across all samples) the ten loci yielded between 15 and 52 alleles, with a total number of 307 alleles. A comparison of genetic diversity over all loci and populations is given in table 3. Global F-statistics detected significant inbreeding (F_{IS}) over all individuals (0.13, $p < 0.001$), using a two-sided test of significance after 1000 permutations.

At the population level values for gene diversity corrected for samples size (H_E) were very high and similar across all populations (0.85–0.86). Such high values are expected for polyploid species, particularly decaploids. Allelic richness (based on 14 individuals) was equally high and similar between all populations (13.93–15.07). Significant but moderate inbreeding coefficients (F_{IS}) were obtained for all populations, see table 3.

Table 2. Monitoring data from twelve *Salix lapponum* and seven *S. myrsinites* sites in the Cairngorms National Park, Scotland, sampled across three estates (Wildland Glenfeshie, Mar Lodge and Abernethy). Data was recorded in 2019.

Species	Site ID	Genetic cluster ID	Elevation (m)	Known plants	Female	Male	Gender not known	Average % browsing damage per plant	Genetic samples taken	Plants with catkins	Juveniles
Salix lapponum	GF1	SL1	850	24	17	3	4	5.42	24	20	1
	GF2	SL1	840	18	7	6	5	4.19	16	15	0
	ML3	SL1	830	5	5	0	0	12.00	4	5	1
	ML4	SL1	655	4	2	0	2	5.00	4	2	1
	ML5	SL1	650	2	1	0	1	2.50	2	1	0
	ML6	SL1	800	5	2	1	2	18.00	5	4	0
	ML7	SL1	910	1	1	0	0	0	1	1	0
	ML8	SL3	750	14	8	4	2	10.61	14	13	1
	ML9	SL4	760	8	4	1	3	33.13	8	6	0
	AB10	SL1	870	6	3	0	3	10.84	6	4	0
	AB11	SL1	880	3	3	0	0	20.00	3	3	0
	AB12	SL2	880	29	10	10	9	10.77	29	21	0
Salix myrsinites	GF1	SM1	580	41	17	15	9	4.10	41	32	3
	GF2	SM1	700	40	21	12	7	4.41	40	33	0
	GF3	SM2	870	29	12	9	8	0.24	29	23	0
	GF4	SM3	850	16	15	1	0	0	16	16	0
	ML5	SM4	840	26	7	13	6	11.15	26	22	0
	ML6	SM5	800	43	27	13	3	1.46	40	40	6
	AB7	SM5	870	1	1	0	0	30.00	1	1	0

Table 3. Genetic variability of ten microsatellite loci estimated for all populations of *Salix myrsinites* and *S. lapponum*.

Pop ID	n	N_A	N_E	H_O	H_E	A_R	F_{IS}
				Salix myrsinites			
SM1	79	23.00 (±2.52)	8.68 (±1.17)	0.74 (±0.05)	0.86 (±0.02)	15.07 (±1.34)	0.15***
SM2	26	18.20 (±1.74)	7.86 (±1.02)	0.77 (±0.06)	0.85 (±0.02)	14.18 (±1.23)	0.10***
SM3	29	18.80 (±1.72)	8.02 (±0.80)	0.75 (±0.05)	0.86 (±0.02)	13.93 (±0.98)	0.13***
SM4	38	21.40 (±2.15)	7.99 (±0.86)	0.76 (±0.05)	0.86 (±0.02)	14.88 (±1.03)	0.12***
SM5	14	15.20 (±1.61)	8.51 (±0.96)	0.80 (±0.05)	0.86 (±0.02)	13.93 (±1.37)	0.09***
Total	186	30.80 (±3.33)	9.70 (±1.19)	0.75 (±0.05)	0.88 (±0.02)	16.69 (±1.37)	0.14***
				Salix lapponum			
SL1	53	10.50 (±2.35)	3.94 (±0.50)	0.56 (±0.07)	0.67 (±0.06)	5.39 (±0.59)	0.16**
SL2	20	4.10 (±1.07)	1.95 (±0.55)	0.43 (±0.09)	0.43 (±0.07)	3.23 (±0.54)	ns
SL3	10	3.50 (±0.79)	2.73 (±0.20)	0.62 (±0.06)	0.58 (±0.04)	3.36 (±0.38)	ns
SL4	8	3.00 (±2.40)	2.14 (±0.77)	0.46 (±0.09)	0.46 (±0.08)	3.00 (±0.81)	ns
Total	91	11.70 (±1.41)	3.39 (±0.51)	0.53 (±0.05)	0.65 (±0.05)	5.08 (±0.65)	0.19***

Abbreviations: n: number of genotyped individuals; N_A: number of alleles; N_E: effective number of alleles; H_O: observed heterozygosity; H_E: gene diversity corrected for sample size; A_R: allelic richness based on 14 individuals for *S. myrsinites* and 8 individuals for *S. lapponum*; ±SE. F_{IS}: inbreeding coefficient; *** $= p < 0.001$ ** $= p < 0.01$, ns = not significant.

3.2.2. S. lapponum

At the species level (across all samples) the 10 loci yielded between 7 and 24 alleles, with a total number of 117 alleles. Global F-statistics detected significant inbreeding (F_{IS}) over all individuals (0.11, $p < 0.001$), using a two-sided test of significance after 1000 permutations.

At the population level values for gene diversity corrected for samples size (H_E) were lowest in SL2 (0.43 ± 0.07 SE, and hereafter) and SL4 0.46 (±0.08) and highest for SL1 (0.67 ± 0.06). Allelic richness was based on eight individuals and similar for SL2, SL3 and SL4 and all lower than SL1 which was $A_R = 5.39$ (±0.59). Significant inbreeding coefficients (F_{IS}) were only obtained for SL1 (0.16, $p < 0.001$). Significant values were also obtained when not clustering all westerly locations merged into SL1, indicating that the significant F_{IS} values for SL1 are not likely to arise from a Wahlhund effect.

The highest average individual kinship coefficient within all analysed *S. lapponum* populations is found in populations SL2, SL3 and SL4 (0.13 ± 0.01, 0.26 ± 0.02, and 0.22 ± 0.02, respectively). The clustered population SL1 had the lowest kinship value, 0.02 ± 0.01 (see figure 2). We also performed the analysis for

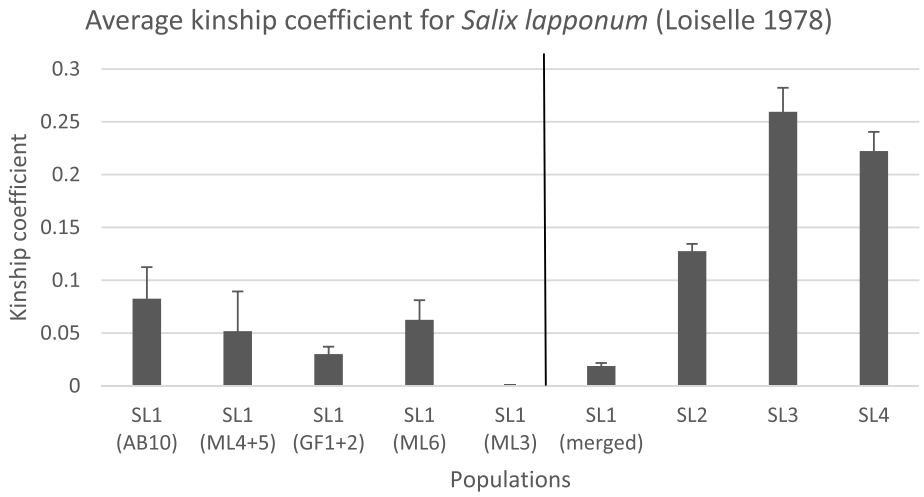

Figure 2. Average kinship coefficients for *Salix lapponum* within four populations (SL1-4), representing four genetic clusters as defined by a STRUCTURE analysis. This population definition groups individuals from different locations into population SL1. Average kinship coefficients from individual locations within SL1 are indicated left of the separation line, except for ML7 and AB11 as these consisted of just a single plant.

individual sites within SL1 to test whether the low kinship in SL1 is representative of all sites. The result show that SL2, SL3, and SL4 still have the highest kinship values and that all the smaller clustered sites within SL1 have on average a lower kinship, see figure 2.

3.3. Genetic differentiation

3.3.1. S. myrsinites

The STRUCTURE analysis identified two genetic clusters for *S. myrsinites*, with the highest ΔK value for K2. This largely delineates populations SM1 from the rest. A further and biologically more meaningful grouping is K5 which divides individuals into five genetic clusters and is in accordance with current geographic location of populations. Only few individuals where assigned to 100% (or near) to a genetic group though, indicating that some gene flow is present across the study area, see figure 3(a).

A principal component analysis (PCA) confirmed a structuring of samples and a delineation of populations SM1 from all other samples, see figure 4.

Global *F*-statistics revealed significant population differentiation over all adults (Jost's *D* value of 0.21 ($p = 0.01$)), using a two-sided test of significance after 1000 permutations. Pairwise genetic differentiation between populations is intermediate. Strongest differentiation is found between population SM3 and all other populations, highest between SM3 and SM2 (0.30) and SM3 and SM5 (0.28). Genetic differentiation between all other populations is 0.16–0.25, see table 4.

3.3.2. S. lapponum

The STRUCTURE analysis identified that the most likely number of genetic clusters is three (showing the highest ΔK value at K3), though further sub-structuring individuals for K4 and K5 into four different populations (SL1-4). No one clustering solution consisted of all individuals being assigned consistently to the same genetic group, indicating gene flow across the study area. Individuals in population SL1 had a similar proportion of two different genetic clusters and consisted of several sample locations, some of which are geographically distant, see figure 3(b). A PCA confirmed the structuring of samples by STRUCTURE and pairwise genetic comparisons, see figure 5.

Global *F*-statistics revealed significant population differentiation over all adults (Jost's *D* value of 0.28 ($p = 0.01$)), using a two-sided test of significance after 1000 permutations. Pairwise genetic differentiation between populations was strongest between SL3 and SL4 (Jost's *D* value 0.42), and lowest between SL1 (merged sites) and SL2 (Jost's *D* value = 0.13), see table 5.

3.3.3. Hybridisation

Within the scope of this study, it was not possible to identify specific alleles that are restricted to pure individuals. Morphological in combination with genetic data was not reliable in identifying hybrids due to morphological variations of the species.

Using ploidy alone, we identified two samples at site AB7 for *S. myrsinites* that were diploid at all markers and are therefore assumed to be a different species. For *S. lapponum* two samples from GF2 and two from

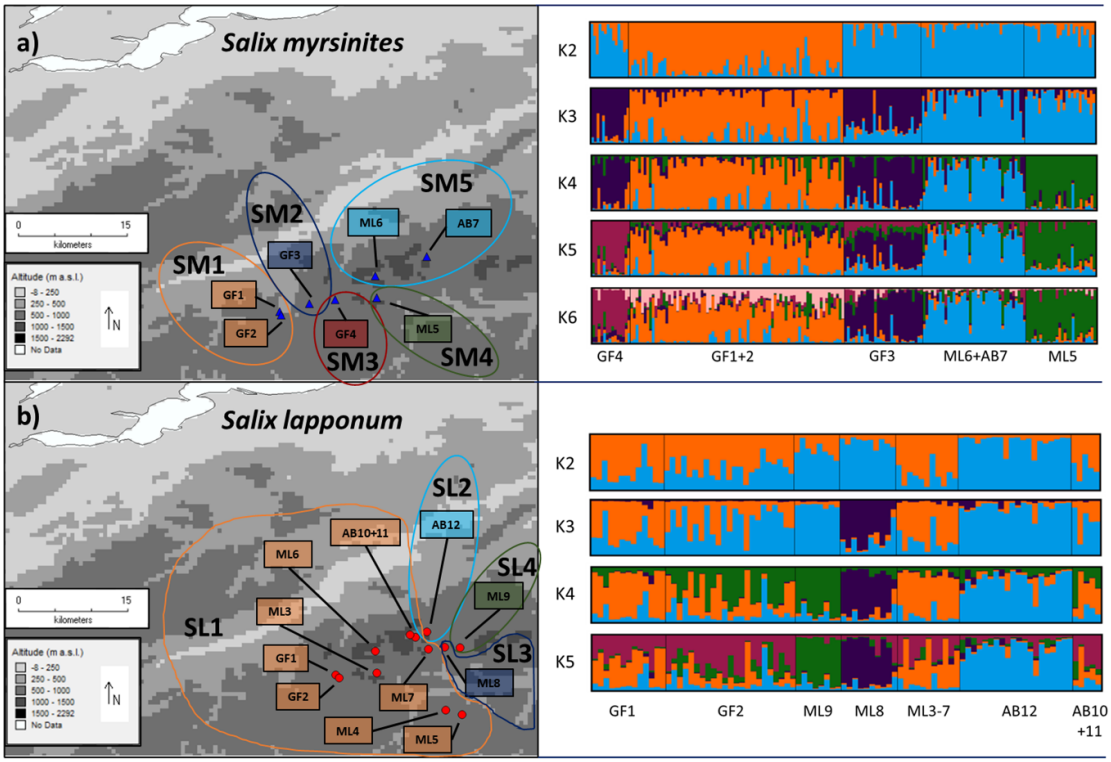

Figure 3. (a) Map indicating sample locations for individual *Salix myrsinites* sites (GF1-AB7) as well as populations according to STRUCTURE genetic grouping (SM1-5). STRUCTURE bar plots for 186 *S. myrsinites* samples for K2 to K6 on the right. Each bar along the *X*-axis represents an individual, assigned to a certain proportion (*Y*-axis) to a genetic cluster. (b) Individual *Salix lapponum* sites (GF1-AB12) and populations according to STRUCTURE (SL1-4). STRUCTURE bar plot for 91 *S. lapponum* samples for K2 to K5 to the right.

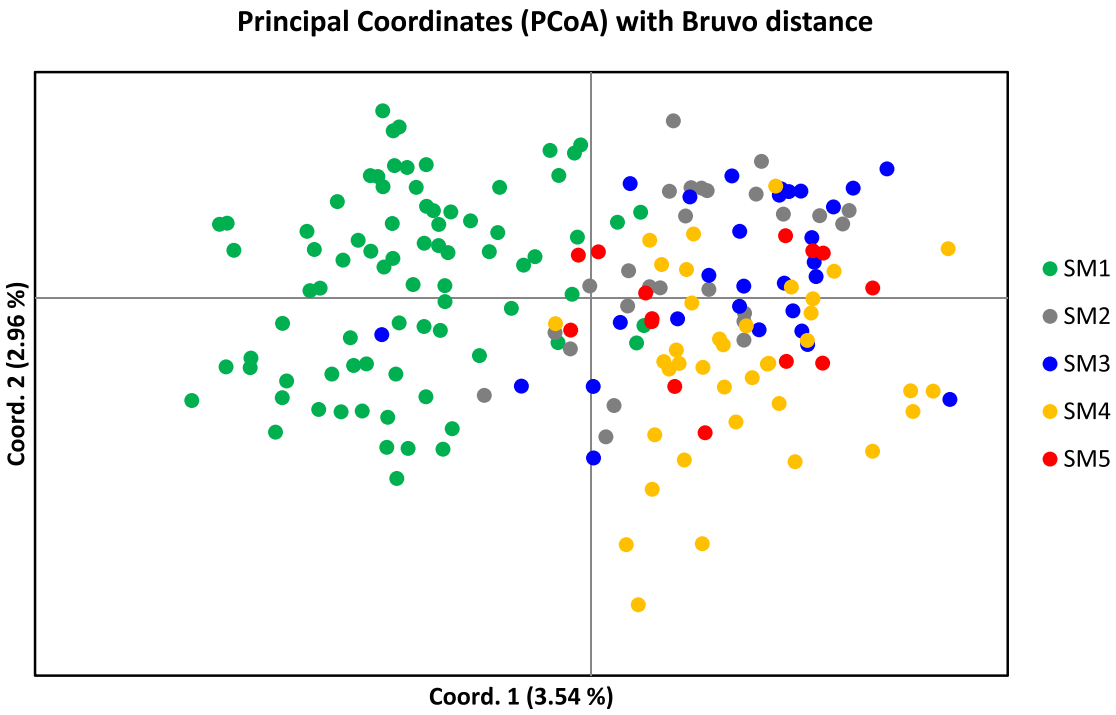

Figure 4. PCoA plot based on genetic distance of 186 *Salix myrsinites* samples and five populations (SM1-5) using Bruvo genetic distance.

Table 4. Pairwise Jost's *D* values between *Salix myrsinites* populations in the Cairngorms National Park. All samples were significant ($p < 0.05$).

	SM1	SM2	SM3	SM4	SM5
SM1	0				
SM2	0.17	0			
SM3	0.25	0.30	0		
SM4	0.18	0.20	0.23	0	
SM5	0.18	0.16	0.28	0.19	0

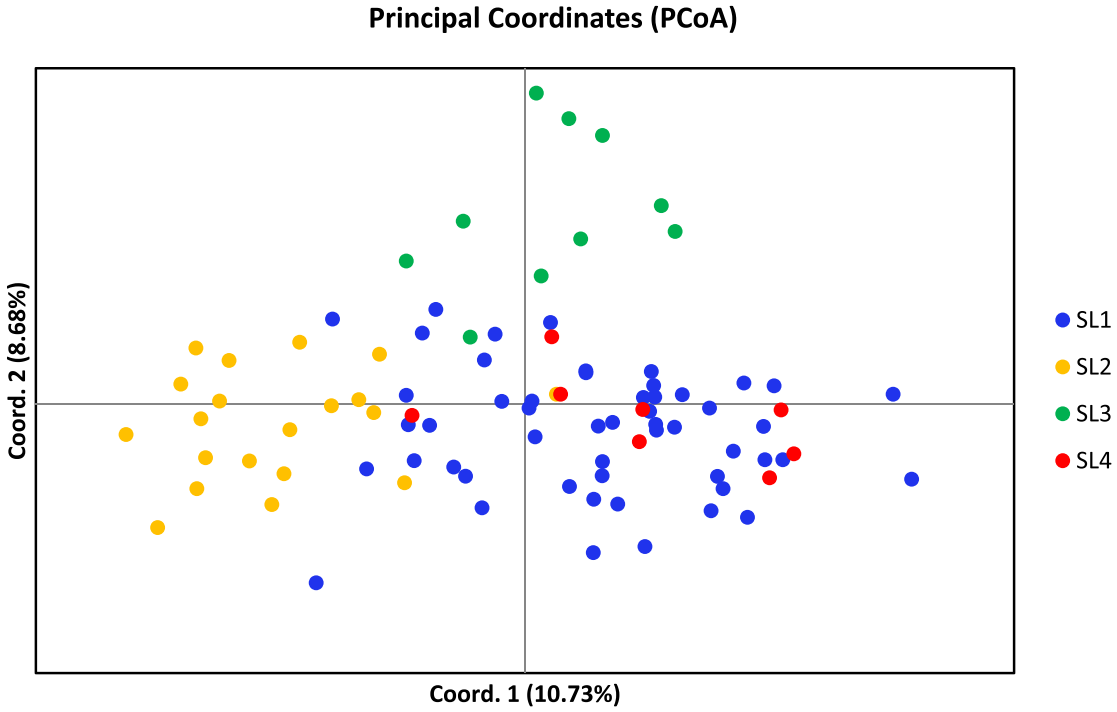

Figure 5. PCA plot based on genetic distance of 91 *S. lapponum* samples and four populations (SL1-4).

Table 5. Pairwise Jost's *D* values between *Salix lapponum* populations in the Cairngorms National Park. All samples were significant ($p < 0.05$).

	SL1	SL2	SL3	SL4
SL1	0			
SL2	0.13	0		
SL3	0.33	0.34	0	
SL4	0.19	0.28	0.42	0

ML6 were polyploid and therefore not pure *S. lapponum*. Many *Salix* species are polyploid and it is not possible to tell from this data what species *S. lapponum* hybridised with or whether these samples came from a different species altogether.

4. Discussion

4.1. Historic gene flow and landscape connectivity

Over the last few centuries ongoing woodland loss in Scotland has led to extremely fragmented habitats. Unless species can maintain long-distance gene flow across areas of unsuitable habitat their populations will remain as isolated patches with all associated negative genetic consequences. Gene flow can counteract these negative consequences (such as inbreeding depression and a loss of genetic diversity) and it is therefore important to understand to what degree populations have been connected in the past and whether contemporary gene flow is likely. Indeed, a microsatellite study on *Crepis sancta* demonstrated that in a fragmented environment connectivity was the main factor decreasing patch extinction (Dornier and Cheptou 2012).

Gene flow is present for both *S. myrsinites* and *S. lapponum* across the studied populations in the Cairngorms National Park. While for *S. myrsinites* genetic structuring is mostly in accordance with their geographic distribution, some *S. lapponum* shrubs cluster into the same genetic group (SL1) even when geographically distant (up to 18 km apart). This could indicate seed or pollen dispersal distances encompassing the whole study area. Only populations located in the northeast (SL2, 3 and 4) formed separate genetic clusters. These populations also had higher kinship values and are therefore likely to be genetically more isolated. Alternatively, the clustering of distinct, scattered shrubs into one genetic group could be due to higher hybridisation rates with other willow species. Individual shrubs are less likely to receive incoming *S. myrsinites* or *S. lapponum* pollen and might be more easily pollinated by other surrounding willow species, though this could not be confirmed with our data and needs further investigation.

There are species capable of maintaining gene flow even in a fragmentated landscape, either through insect pollination (e.g. Finger *et al* 2014, González-Robles *et al* 2021) or wind-dispersed seeds (Bacles *et al* 2006). A study on Scottish *Fraxinus excelsior* showed that while long distance pollen exchange between fragmentation is present, the dispersal distance is often driven by population size. Larger populations predominantly act as pollen donors whereas smaller ones are predominantly pollen recipients (Bacles and Ennos 2008), highlighting the importance of maintaining large populations in a landscape. For montane willows in Scotland, such large populations are not present. As only adult individuals have been analysed in this study, the observed genetic structuring represents historic rather than contemporary gene flow. The age of the sampled shrubs is not known but could possibly be over 100 years old. They are though unlikely presenting a less fragmented environment as habitat fragmentation in Scotland has accelerated in the 16th century due to transhumance style grazing (Oosthoek 2013). To analyse contemporary gene flow and explore whether gene flow rates have changed a genetic analysis on seedlings or seeds is required (González-Robles *et al* 2021). It is therefore possible that while historic gene flow is present, contemporary populations have become genetically isolated or too small to attract pollinators.

4.2. Genetic diversity and inbreeding in montane willow populations

Genetic diversity (heterozygosity and allelic richness) in remaining shrubs is high and similar between populations within both species and particularly high for *S. myrsinites* which is expected as the species is decaploid (Meirmans *et al* 2018, Karbstein *et al* 2021). Genetic diversity detected in a previous study on *S. lapponum* in Corrie Sharroch using microsatellites was even higher (expected heterozygosity $H_E = 0.70$) compared to our results ($H_E = 0.65$) (Stamati *et al* 2007). We cannot exclude that some of the overall detected genetic diversity for both species is in part due to hybridisation with more widespread willow species, though observations in the field suggest that many hybrids are not as fit (in terms of survival and plant vigour) as pure shrubs and therefore less likely to contribute much to the gene pool.

Due to the extensive degree of hybridisation in *Salix* and the lack of species specific alleles detected with the set of microsatellite loci used in this study, it is likely though that some hybrids have not been identified (Stace 2019). Nevertheless, the maintenance of a high heterozygosity and allelic richness will have positive effects on plant fitness and provide valuable source material for conservation translocations (Reed and Frankham 2003, Leimu *et al* 2006). Moreover, hybrids can have an evolutionally advantage relative to their parents, especially in changing environments and can occupy more extreme habitats, a has been demonstrated for two hybrid populations between *Salix purpurea* and *Salix helvetica* in the European Alps (Gramlich *et al* 2016). As such hybrids could potentially have an important role in restoration.

Clonal reproduction is restricted for both species and therefore the observed number of shrubs represents the census populations size, confirming previous findings for *S. lapponum* (Stamati *et al* 2007). Nevertheless, genetic diversity, in terms of demographic diversity is extremely small for both species, particularly for *S. lapponum* with only two locations containing 20 and 23 shrubs and most others less than 10 shrubs. *S. myrsinites* populations are slightly larger (largest 79 shrubs) but most still consist of less than 38 shrubs. Skewed sex ratios further reduce the effective population sizes as both species are dioicous. Some populations (seven out of thirteen locations) will struggle to reproduce without incoming pollen as they only consist of female shrubs, something also observed for *Salix lanata* in Scotland (Marriott *et al* 2015).

Significant but intermediate levels of inbreeding were detected in both species. For *S. lapponum* inbreeding was only detected in SL1. To test whether this was due to ignoring sub structuring (Wahlhund effect) from clustering distant populations we repeated the analysis using populations according to their geographic locations. Inbreeding values were still significant and derived mainly from location GF1, indicating that in this location reproduction is mainly within population. Current small population sizes, the very low numbers of juveniles found in populations despite reduced grazing pressure, as well as low observed seed set in the field for both species, provide first signs of negative impacts on fitness for the next generation, either through low pollen quantity, inbreeding depression, or a combination of both.

4.3. Conclusions for restoration and population management

All analysed populations in this study are at an immediate risk of being lost. Some populations consist of just single male or female shrubs and natural reproduction even within larger populations has seldom been observed. Urgent conservation action is required to increase reproductive success in Scottish populations. Genetic rescue, e.g. through the introduction of new individuals into small and isolated populations, can help to secure long-term resilience of populations and can thus help to stem biodiversity loss (Whiteley *et al* 2015, Bell *et al* 2019). The risk of outbreeding depression is low for *S. myrsinites* and *S. lapponum* due to the historic gene flow detected across the study area. Anyway, the benefits from heterosis will most likely outweigh outbreeding depression after a few generations (Willi *et al* 2007) and natural selection will reduce survival of less adapted genotypes in the long term (Sgro *et al* 2011).

To increase numbers of individuals for *S. myrsinites* and *S. lapponum* and to create safe sites in case natural populations are lost, existing populations should be reinforced as well as new populations established with a mix of male and female plants nearby existing populations. This will create stepping-stones for gene flow between existing populations. Reinforced and new populations will have to be large enough (possibly hundreds of plants) to ensure genetic health, resilience to grazers, competitive advantage, and also to attract pollinators (Abeli *et al* 2016). Such demographic rescue will help the immediate risk of extinction by reducing the potential impact of stochastic effects.

To achieve genetic rescue, reinforced or new populations should consist of unrelated individuals and have a high genetic diversity to provide populations with the basis to being able to adapt to environmental change, or resilience to other environmental stresses (Engelhardt *et al* 2014). To allow populations to persist as self-sustaining populations in the long-term, gene flow between populations needs to be (re-)established by creating new populations in pollination or seed dispersal distance. Distance between populations may have to be fairly short to allow for gene exchange through pollen flow. For *S. lanata* which is pollinated by mainly bumblebees and flies, a distance of at most 50 m has been suggested to allow successful reproduction through gene flow (Marriott *et al* 2015). As genetic diversity is high for both species, and historic gene flow present, all populations are suitable as source populations, particularly when mixed to avoid inbreeding depression in restoration sites. Genetic rescue has already been demonstrated to help rare, plant species in Scotland. For example, the clonal and self-incompatible *Linnea borealis* was unable to reproduce in its remaining patches and genetic rescue (through conservation translocations) was the only way to overcome reproductive isolation (Wiberg *et al* 2016).

For restoration to be successful, it is key that the ecological conditions are suitable. This may require low plant competition, open suitable ground for germination, suitable soil conditions and the right microclimate (de Vere *et al* 2009, Sedlacek *et al* 2014, Little *et al* 2016). It is possible that seed establishment is limited at altitudes higher than the current distribution range and that expansions uphill may be impeded due to novel soil microbial communities as has been shown for *S. herbacea* in Switzerland (Sedlacek *et al* 2014). Low grazing pressure is likely to be critical and this is often hard to achieve in Scotland. Deer are an important herbivore, but voles and mountain hares can also be significant browsers. Unless grazing pressure is reduced to a sustainable level, expensive and often ineffective fencing will be necessary to protect plants (Scheidel and Bruelheide 2001, Fenu *et al* 2016). Fencing is though not desirable due to impacts on access, landscape and wildlife within the National Park. Snow cover is also important for protecting willows at times when they are likely to be browsed but warming winters may mean that this winter protection has been reduced. Climate change will therefore likely further affect the species persistence in the landscape and could require further conservation measures.

Data availability statement

All data that support the findings of this study are included within the article (and any supplementary information files).

Microsatellite sequences: Genbank accessions ON676485-95

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Author contributions

All authors contributed to the study conception and design. A F performed the research and analysed the data. A B, B D, S R collected population data and samples. S R, T M, N C provided samples. A F, S R and N C wrote and revised the paper. All authors approved the final manuscript.

Conflict of interest

The authors have no competing interests to declare.

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