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Long-term change and genetic connectivity in species-rich mesotrophic grassland vegetation with particular reference to hay meadows

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Long-term change and genetic connectivity in species-rich mesotrophic grassland vegetation with particular reference to hay meadows

Summary report of findings from a PhD research project

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Summary

Species-rich grasslands have declined significantly and many now have statutory protection as Sites of Special Scientific Interest (SSSIs) and are managed through agri-environment scheme (AES) agreements. Surveys of mesotrophic (neutral) grasslands were undertaken by the Nature Conservancy Council in the 1980s and 1990s, and in the first part of this research project these surveys were repeated in 2012-2014 in the Forest of Bowland in North West England to investigate long-term change in grassland vegetation.

Analysis of the resulting data showed that overall, community composition in 35 grassland sites had not changed. More detailed analysis of a subset of 14 hay meadow sites did reveal a change in composition. Site richness had declined but there was no significant difference in percentage cover of a suite of positive meadow indicator species, and negative indicator species had declined. However, there were changes in individual indicator species, such as decreases in *Alchemilla spp.* and increases in *Euphrasia spp.*, and the vegetation had become more homogenous.

Species-rich hay meadows have a fragmented distribution, and the second part of the study investigated the levels of genetic diversity and connectivity within and between hay meadow plant populations – with *Rhinanthus minor* as the study species.

Leaf samples were collected from meadows in the Forest of Bowland and in Worcestershire with the aim of comparing genetic variation and gene flow in an upland and a lowland region of England. Samples were also collected from grassland sites outside the protected meadows in both regions to assess whether other ‘intermediate’ sites played a role in an ecological network. DNA was extracted from the leaves and analysed using molecular markers (microsatellites).

The results showed that there were similar, moderate levels of genetic diversity in the meadow populations of *R. minor* in Bowland and Worcestershire but that levels of gene flow were limited in both regions and more particularly in Worcestershire, where land-use is more intensive. Connectivity between meadows and intermediate sites was also limited, but again, this was more pronounced in Worcestershire.

The study showed that conservation appears to be being effective in maintaining the meadow community and genetic diversity in *R. minor,* but the current management regime may not be preventing reductions in some key species or the development of more homogeneous vegetation. This could be linked to the application of standard management prescriptions at the regional level.

Landscape-scale conservation of grassland sites should consider of the role of non-agricultural and less species-rich grasslands in an ecological network to maintain gene flow. This may be particularly important in areas with more intensive land-use where enhancement and creation of existing and new sites could be required to boost resources for pollinators. Further research on gene flow in other meadow species including perennials and wind pollinated plants would be of value in gathering evidence for effective landscape-scale conservation.

Acknowledgements

The authors wish to thank the farmers and landowners who kindly provided access to the study sites used in this research. They would also like to acknowledge the valuable advice and support given by the Forest of Bowland Haytime team and the Worcestershire Wildlife Trust. The study would not have been possible without funding from Edge Hill University, Natural England, the Botanical Society of Britain and Ireland and the Natural History Museum’s Botanical Research Fund.

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1. Introduction

There has been a drastic decline in species-rich mesotrophic (neutral) grasslands in the UK since the middle of the 20th century, mainly due to agricultural intensification (Hodgson and others 2005). Mesotrophic grasslands can support a wide range of vegetation communities of conservation importance. Many of the most diverse examples have statutory protection as Sites of Special Scientific Interest (SSSIs) with some sites also designated as Special Areas of Conservation (SACs) under the EU Habitats Directive (Natural England 2008). Management agreements made through the adoption of agri-environment schemes (AES) also play an important role in their conservation management.

This report summarises research undertaken for a PhD project which investigated two aspects of grassland vegetation: (1) long-term change in mesotrophic grasslands and (2) genetic diversity within, and connectivity between, hay meadow plant populations. The long-term change study was undertaken in the Forest of Bowland in North West England and included mesotrophic pastures and hay meadows – with meadows being studied in greater detail. The genetic diversity and connectivity study was carried out in both the Forest of Bowland and in Worcestershire and so included both upland and lowland meadows, with *Rhinanthus minor* as the study species.

The main aim of the long-term change study was to analyse change in community composition in species-rich grasslands since the Nature Conservancy Council (NCC) carried out detailed vegetation surveys mainly in the mid-late 1980s but with a smaller number in the 1990s (Blackstock and others 1999). The NCC survey data provided a valuable baseline prior to the introduction of AESs and, in the case of the Forest of Bowland sites, the surveys also pre-dated the notification of grassland SSSIs in the area. The detail included in the NCC surveys enabled a re-survey of the grassland sites to be undertaken and allowed for an analysis of differences between the ‘before’ and ‘after’ surveys. The results were used to draw conclusions about change since statutory protection and agri-environmental management measures were introduced.

Species-rich grasslands, including hay meadows, now have a very fragmented distribution and the focus of their management, along with that of other semi-natural habitats, is shifting from a site-based approach to one which also considers the landscape-scale (Donaldson and others 2017; Lawton and others 2010). Concerns have been raised that small and isolated populations may be at risk of reduced genetic diversity due to limited population sizes and lack of gene flow between populations (Leimu and others 2010; Young and others 1996). The study of genetic diversity and connectivity in populations of a key meadow species, *R. minor*, was designed to analyse levels of genetic variation and gene flow in meadows in two contrasting regions of England, and to consider the implications for meadow conservation. This part of the study included protected meadow sites and ‘intermediate’ grasslands such as roadside and footpath verges and non-protected agricultural grasslands.

This report summarises the main methods and results of the two investigations and discusses the findings in the context of conservation. The research addressed the following questions:

- How has the community composition of mesotrophic grassland vegetation in the Forest of Bowland changed over the past 25 years?
- Is the pattern of change influenced by the management regime, and in particular by hay meadow management?
- Has the fragmented distribution of hay meadows affected genetic diversity within, and gene flow between, populations of a key meadow species, *Rhinanthus minor*?
- How do levels of genetic diversity and connectivity compare across meadows and other grassland fragments in an upland (Forest of Bowland) and lowland (Worcestershire) region of the UK?

2. Methods

Principal methods used in the study:

- Surveys undertaken by the Nature Conservancy Council in the 1980s and 1990s were repeated in 35 grassland sites in the Forest of Bowland in north-west England;
- Change in overall community composition, and species 'winners' and 'losers' were analysed;
- Detailed analyses of the survey results in 14 species-rich hay meadows were undertaken;
- Leaf samples were collected for DNA analysis from *Rhinanthus minor* in meadows and other grassland sites in the Forest of Bowland and Worcestershire;
- DNA was extracted and analysed using molecular markers (microsatellites);
- The genetic data were used to investigate levels of genetic diversity and gene flow in *Rhinanthus minor*.

2.1 Methods for the long-term change study

2.1.1 Study area

The study sites for the long-term change study were located within an area of approximately 450 km^2 in the valleys of the Forest of Bowland which is an upland area in north-west England (Fig. 1). The mean annual precipitation for the region is 1294mm, mean January temperature is 4.0°C and mean July temperature is 15.8°C (Met Office 2016).

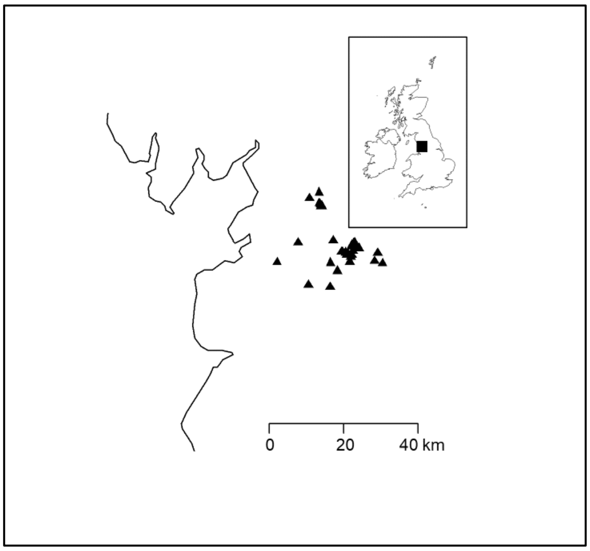

Fig. 1 Location map for the long-term change study sites

2.1.2 Site selection

The study focused on sites which had been surveyed by the Nature Conservancy Council (NCC) in the 1980s and 1990s (Blackstock and others 1999). 35 mesotrophic grassland sites were identified in the Forest of Bowland for which survey records were available.

Of the 35 sites in the survey:

- 14 had been managed continuously as hay meadows;
- 10 had been managed by grazing (cattle, sheep or a mixture of both);

- 6 sites were described as hay meadows at the time of the NCC surveys but had seen a change to more intensive management (cut for silage or heavily grazed);
- 5 sites appeared to be unmanaged or undermanaged at the time of the second survey.

Table 1 gives the details of the sites and the numbers of quadrats recorded in the survey. Plates 1 and 2 show examples of a grazed site and a meadow.

Table 1 Site details for mesotrophic grasslands surveyed in the Forest of Bowland

Site ID	Management type	Size (ha)	Altitude (m asl)	No. of quadrats
AM	former meadow	1.8	185	3
BG	meadow	5.47	180	1
BG2	meadow	2.2	180	1
BG3	meadow	3.1	180	2
BS (3 fields)	meadow	7.65	150	12
BS1	meadow	2.3	180	2
BS3	grazed	1.2	170	3
CB	meadow	0.54	60	4
DH	meadow	0.4	190	2
FH	meadow	1.63	105	2
FHM	meadow	3.33	210	4
HHL	unmanaged	10.3	195	6
HHM	unmanaged	0.3	105	2
LBL	former meadow	1.7	140	2
LCB	grazed	6.0	180	3
LCM	meadow	5.26	190	2
LHBS	grazed	0.76	130	2
LHG	grazed	2.2	100	3
LRS	unmanaged	0.2	120	2
LSM	former meadow	1.1	230	1
LWM	unmanaged	3.6	105	2
MM (2 fields)	meadow	9.09	155	9
MM2	grazed	0.7	160	3
NI	meadow	2.09	125	6
NKM	grazed	3.9	180	6
OWP	grazed	0.3	160	6
PHB	unmanaged	0.5	135	2
PP	grazed	1.8	150	10
RH	former meadow	1.8	80	2
SFP	grazed	4.5	230	11
SM	meadow	3.63	200	2
SPM	grazed	1.4	280	6
TB (5 fields)	meadow	11.87	155-180	7
TL	former meadow	0.4	220	2
TSM	former meadow	6.4	185	3

m. a. s. l = metres above sea level, ha = hectares

Plate 1 Example of a grazed site

Plate 2 Example of a meadow site

The majority of the NCC surveys took place before the UK National Vegetation Classification (NVC) was published but the survey records did refer to the proposed NVC classifications and identified the main communities as: upland hay meadows MG3 *Anthoxanthum odoratum-Geranium sylvaticum*, floodplain meadows MG4 *Alopecurus pratensis-Sanguisorba officinalis* and lowland hay meadows or pastures MG5 *Cynosurus cristatus-Centaurea nigra* communities (Rodwell 1992).

2.1.3 Survey methods

The repeat surveys (described in this report as the **second survey**) were carried out using the original methods in the summers of 2012 - 2014. The original surveys (the **first survey**) followed the NCC's guidance and involved 1m x 1m quadrats and the compilation of a site species list. Presence and abundance of all vascular plants were recorded using the Domin scale. The number of quadrats varied depending on the size and complexity of the sites (Table1). The species list covered all areas of the site on and within the site boundary.

2.1.4 Data analysis

An exploratory approach to data analysis was taken for the 35 grassland sites because a random sampling approach for the collection of species list data and may have been difficult to apply in grazed sites which often had quadrats in small vegetation stands such as flushes. A more detailed analysis was undertaken for the meadow sites data where it was assumed that the stratified random sampling method described in the NCC survey guidance (Smith and others 1985) would have been straightforward.

Data analysis is described in detail in two of the published articles which accompany this PhD research (Sullivan and others 2017; Sullivan and others 2018) and in the PhD thesis which is available to download (Sullivan 2018). A summary of the methods used is as follows:

- Change in overall community composition in the 35 sites was analysed using non-metric multidimensional scaling (NMDS). This method was used to analyse both the quadrat survey data and the species list data and was applied to the four management types listed in section 2.1.2 above.
- Differences between the quadrat survey data and species list data were investigated using Procrustes analysis.
- Species losses and gains were analysed by ranking species according to the frequency at which they had been recorded in each site.
- For the 14 meadow sites change in community composition was investigated using redundancy analysis, and a permutation test was undertaken to assess significance.
- Change in site species richness was tested by a paired *t*-test.
- Patterns in the diversity of the meadow community vegetation were analysed by testing for multivariate homogeneity.
- Analyses of percentage cover of positive and negative indicator species for hay meadows were carried out. A list of indicators was drawn up from the JNCC's *Common Standards Monitoring Guidance for Lowland Grasslands* (JNCC, 2004) and a study of upland and lowland meadows by Kirkham and others (2014). Difference in percentage cover of positive and negative species between the two survey years was investigated using a Wilcoxon rank sum test. The indicators used in the study are listed in Appendix 1.
- Analyses of Ellenberg Indicator Values (EIVs) were undertaken to give an indication of change in soil conditions. These were using permutation tests on unweighted mean Ellenberg indicator values for soil moisture (EIV moisture), pH (EIV pH) and fertility (EIV fertility) using the method developed by Zelený and Schaffers (2012) which was designed to overcome the bias associated with analysis of EIVs. Wilcoxon signed rank tests were used in a comparison of the mean EIVs of the most influential species in the RDA analysis. The permutation test could not be used in this case because two different sets of species were being compared.

2.2 Methods for genetic diversity and connectivity

2.2.1 Study sites and sampling method

Samples of *R. minor* leaves were collected from nine meadow sites in the Forest of Bowland and nine meadows in Worcestershire. Sample sizes and site details are shown in Table 2 and the sampling methods are described in detail in Sullivan (2018) and in the published article which accompanies this part of the research (Sullivan and others 2019). Most of the meadows are SSSIs and all are managed for field dried hay with a late hay cut and no applications of artificial fertiliser. Samples were also collected from other grassland sites in both study areas in order to assess whether populations outside the main meadow sites were also part of an ecological network. These sites, described as 'intermediate sites', included roadside verges, a churchyard and some moderately species-rich agricultural grasslands. See maps in Figs 2 and 3

Table 2 Details of the Bowland and Worcestershire sites used in the genetic diversity and connectivity study

Bowland sites	Altitude (m. a.s.l)	Size (ha)	*N*	Nearest protected meadow
BG	180	5.47	21	1.97
BS	150	7.65	88	0.52
CB	60	0.54	12	3.49
FH	105	1.63	20	3.49
FHM	210	3.33	21	0.46
LCM	190	5.26	18	0.46
MM	155	9.09	40	1.29
NI	125	2.09	12	6.1
TB	155-180	11.87	22	11.59
Worcestershire sites	**Altitude (m. a.s.l.)**	**Size (ha)**	***N***	**Nearest protected meadow**
BA	35	1.07	27	1.6
BC	20	6.84	32	1.3
EM	85	7.0	32	2.2
HW	15	11.41	32	0.8
KP	75	3.91	32	2.4
LM	50	5.12	32	2.6
SA	90	4.67	32	3.1
SB	45	2.21	30	1.9
SW	55	1.75	32	1.50

m. a. s. l = metres above sea level; ha = hectares; *N* = number of samples; Nearest protected meadow = closest protected meadow site in km.

2.2.2 DNA analysis

The leaf samples of *R. minor* were stored in silica gel prior to DNA extraction. DNA analysis was carried out using molecular markers (microsatellites) developed for *R. minor* (Houston and Wolff 2009) and *R. angustifolius* (Ducarme and others 2008). Details of the laboratory methods used to extract and analyse the *R. minor* DNA are described in Sullivan (2018) and Sullivan and others (2019).

Data analysis

Details of the data analysis used for the genetic diversity and connectivity study are described in detail in Sullivan (2018) and Sullivan and others (2019). A summary of the methods is as follows:

- Genetic diversity in the meadow sites was estimated by calculating expected heterozygosity (H_e) by site (population) and for the dataset as a whole (region) and tests for difference between the Bowland and Worcestershire results were performed using a Wilcoxon rank sum test.
- The relationship between meadow site size in hectares and genetic diversity (expected heterozygosity) was tested using a Spearman Rank correlation test.
- The inbreeding coefficient (F_{IS}) was calculated.
- Population differentiation was analysed using F_{ST} values for each population, along with a global estimate of overall genetic differentiation.
- The spatial genetic structure of the meadow and intermediate site populations was explored by performing a spatial principal components analysis (sPCA) (Jombart and others, 2008). Colorplots are used to display the results graphically, and use the red green blue (RGB) colour channel to highlight genetic differentiation with similar colours representing genetic similarity.

Data analysis was carried out in R version 3.3.2 (R Core Team, 2016).

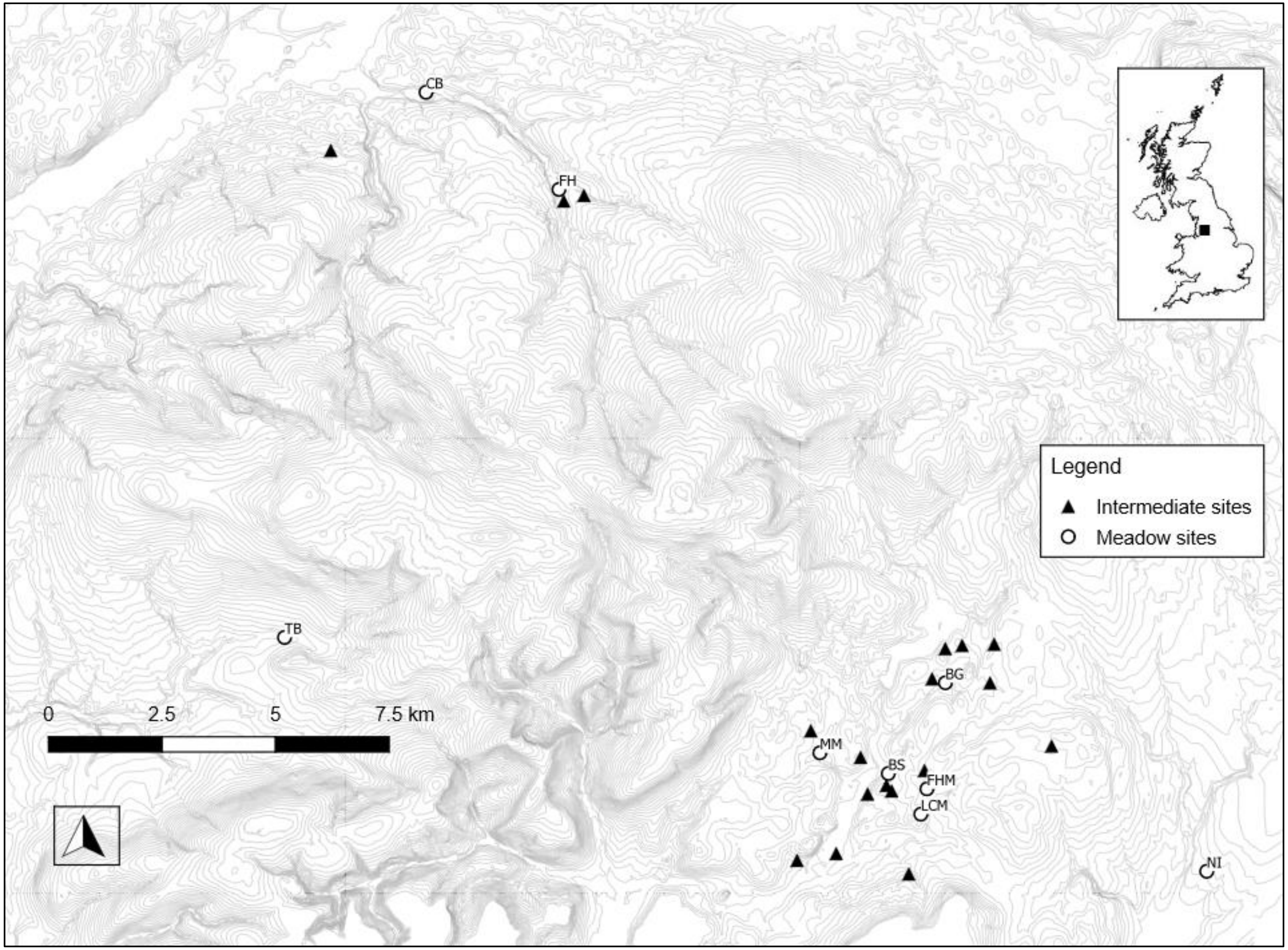

Fig. 2 Locations of Bowland meadow and intermediate sites for genetic diversity and connectivity study.

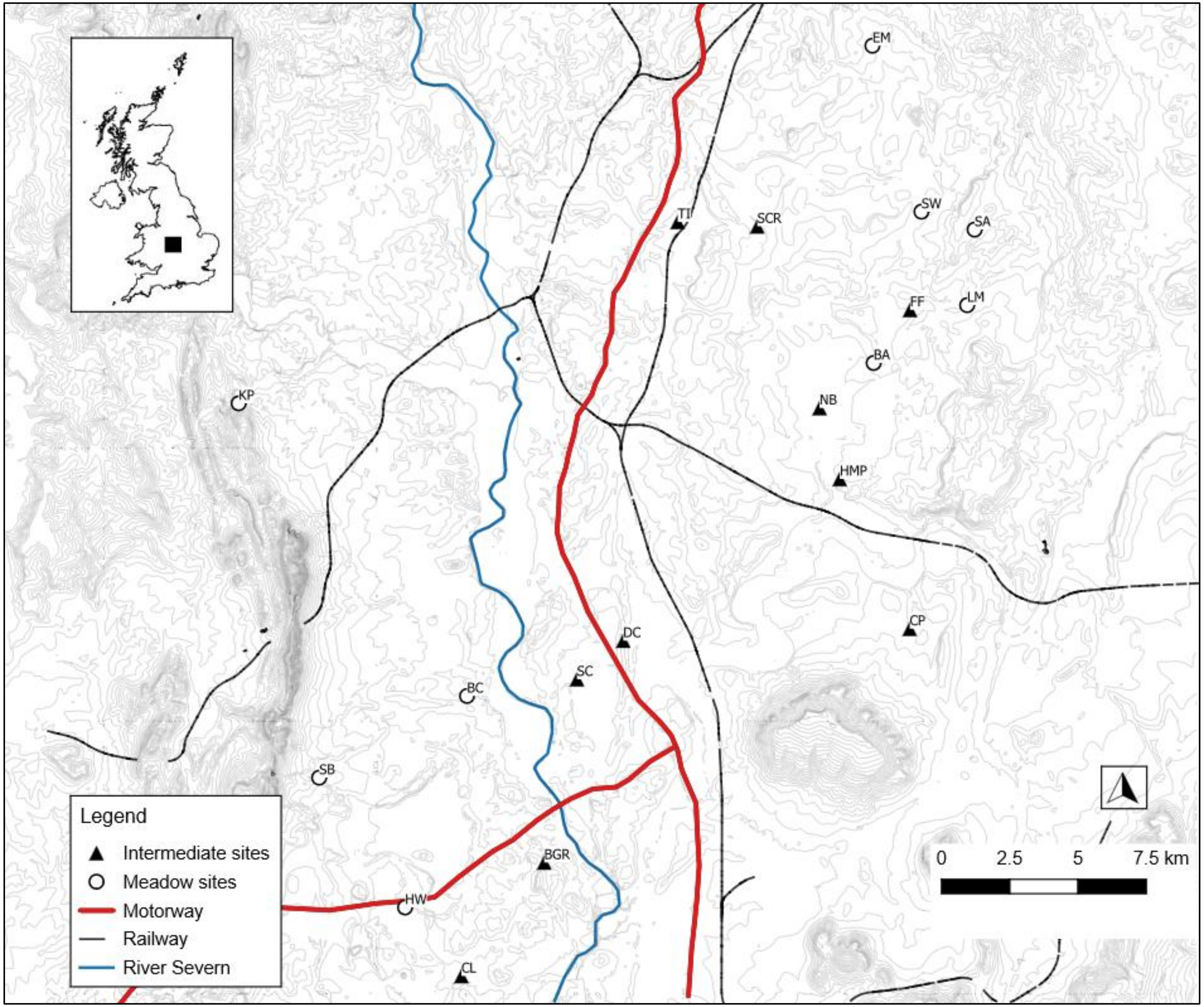

Fig. 3 Locations of Worcestershire meadow and intermediate sites

3. Results

Key results:

- There was little difference in overall community composition between the original and repeat surveys across the 35 sites;
- Quadrat surveys and site species lists picked out different plant communities;
- There was a significant difference in community composition in the 14 meadow sites when the original and repeat surveys were analysed;
- Cover of a suite of positive meadow species was similar in both surveys but individual species showed differences and negative indicator species had declined;
- The meadow vegetation had become more homogenous;
- There were similar levels of moderate genetic diversity in populations of *Rhinanthus minor* in the Forest of Bowland and Worcestershire;
- There was less gene flow in Worcestershire than in Bowland;
- Genetic connectivity between meadows and intermediate grassland sites was limited in both regions but was particularly limited in Worcestershire.

3.1 Long-term change study

In the quadrat survey for all of the 35 sites the total number of species recorded was 152 from the first survey and 144 from the second survey (a decrease of 5.3%). In the species list survey the totals were 268 from the first survey and 229 from the second survey (a decrease of 14.6%).

The NMDS ordination plots do not show a distinct separation of survey sites by year for either the quadrat data or the species list data (Fig 4) indicating that there is little difference in overall community composition between the two survey years.

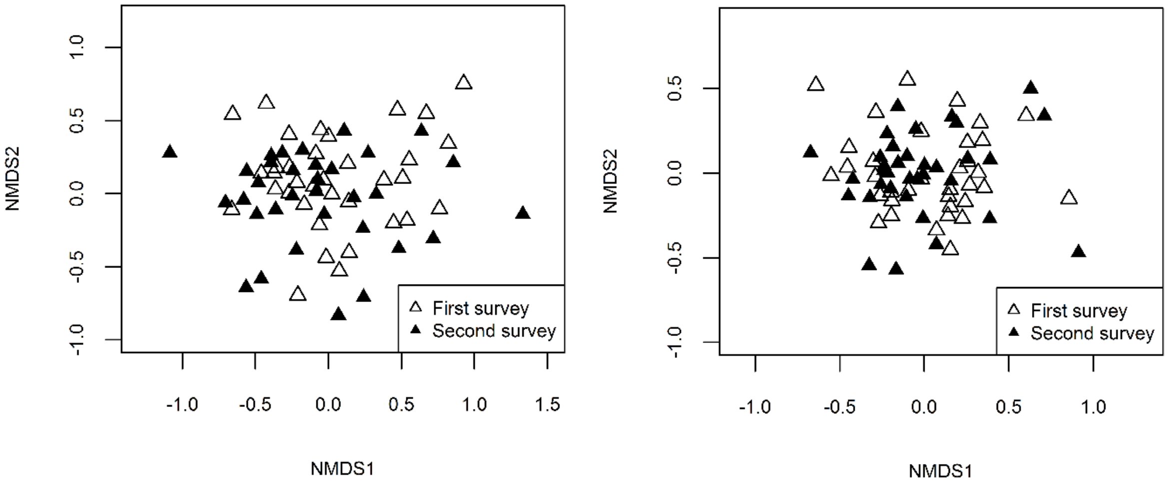

Fig. 4 NMDS ordination of quadrat data (**left**) and species list data (**right**) for the first and second surveys. Points represent grassland sites.

When the NMDS ordinations for quadrat survey and species list survey data were compared the Protest permutation test for the Procrustes analysis returned an *r* value of 0.27 which suggested that there was little correlation between the two ordinations. This result indicates that the two survey methods revealed contrasting results in terms of community composition.

Analyses of the quadrat data by management type showed a change in community composition between the sites formerly managed as meadows and now managed more intensively (Fig 5c). This was expected but Figure 5a also shows some separation between the points representing the two surveys which indicates differences in the community composition of the sites managed consistently as meadows.

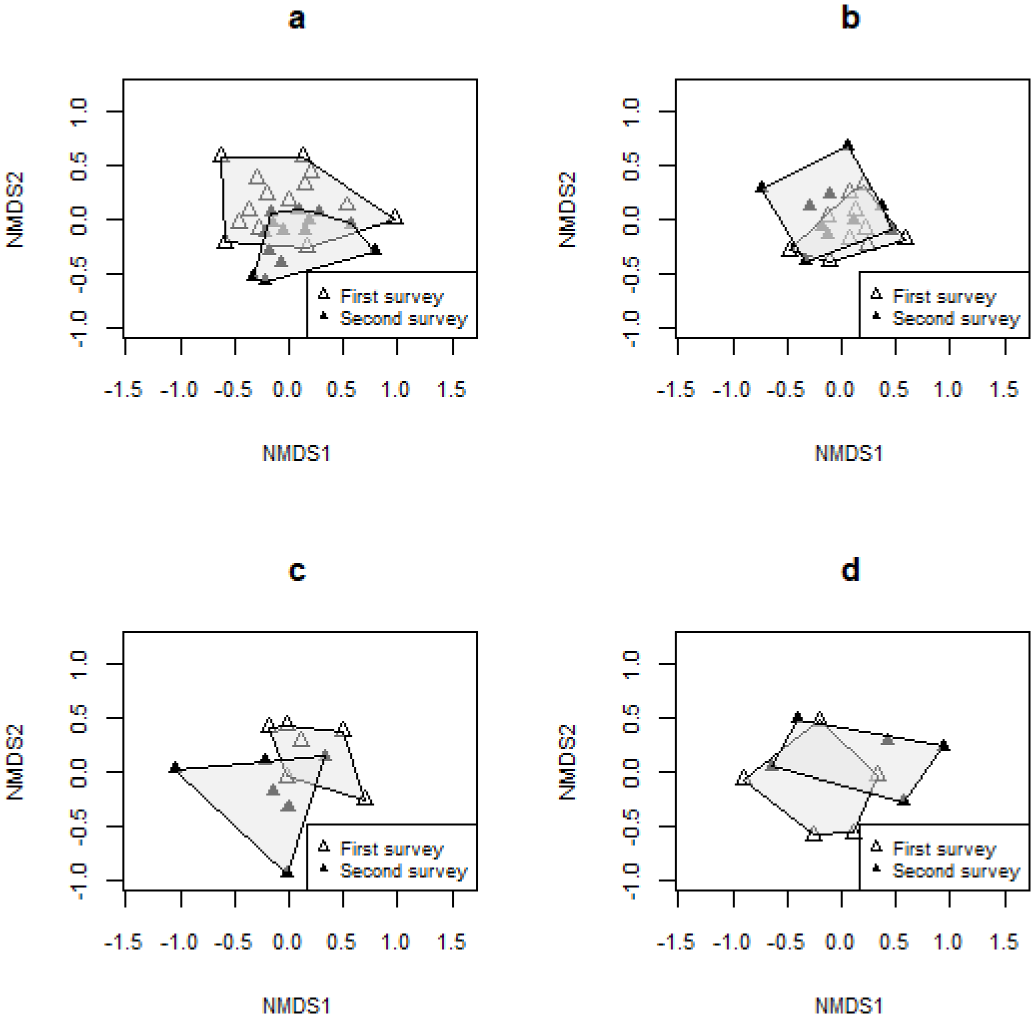

Fig. 5 NMDS plots showing community composition by management type in the two surveys. a = meadows; b = grazed sites; c = changed sites (former meadows); d = unmanaged sites

More species had shown a decrease than an increase in terms of the number of site records (see Appendix 2) for both the quadrat and species list data. 11 of the 25 most decreased species appear in both the quadrat and species list data. Species of conservation importance which had shown a decrease in site records included *Alchemilla spp*, *Conopodium majus* and *Leontodon hispidus*). However species which would be regarded as less favourable for species rich grasslands had also decreased such as *Dactylis glomerata* and *Phleum pratense*. Fewer species had shown a substantial increase in site records, particularly in the quadrat data. Increased species which would be indicative of favourable grassland condition included *Euphrasia spp*. However, species such as *Juncus effusus* and *Urtica dioica* had also seen an increase in site records.

In the detailed analysis of the meadow sites 95 species were recorded across the 14 sites in the first survey whereas 79 species were found in the second survey. Mean site richness was significantly lower in the second survey (27.64, standard error = 2.36) than it was in the first survey (31.71, standard error = 2.78). Test statistics for the paired *t*-test for difference in mean site richness were *t* = 3.32, *P* = (0.005).

Results of the redundancy analysis (RDA) showed that there was a significant difference (*P* = 0.003) between the community composition of the meadow sites in the first survey compared with that of the second survey and the ordination plot in Fig 6 shows some separation by survey year.

The species scores for the first axis were extracted from the RDA output and were ranked according to their magnitude. The ten species most associated with the first survey and the ten species most associated with the second survey are shown in Table 3. Table 3 shows whether these species increased or decreased in terms of the frequency of site records. Positive and negative meadow indicator species are also identified. Some positive indicators decreased such as *Conopodium majus* and *Alchemilla glabra* whilst others increased such as *Euphrasia species*, *Scorzoneroides autumnalis and Rhinanthus minor.* There were also decreases in some negative indicator species, eg *Bromus hordeaceus, Phleum pratense* and *Lolium perenne*, whilst others such as *Ranunculus repens, Juncus articulatus* and *Holcus lanatus* saw an increase. There were increases in widespread grassland species such as *Plantago lanceolata* and *Trifolium pratense*.

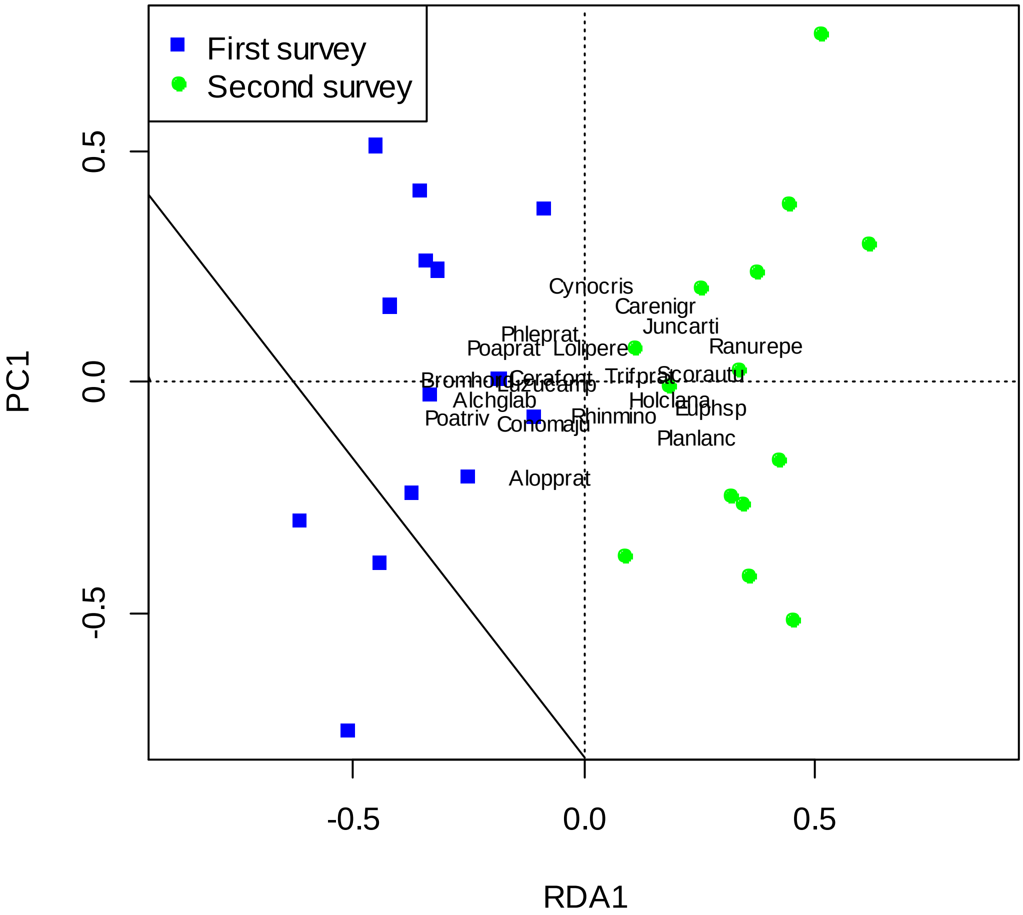

Fig. 6 RDA ordination plot of meadow sites constrained by year. Square symbols show sites in first and second survey. Ten species most associated with first and second survey are shown. For abbreviations please see Appendix 3

Table 3 Species with ten lowest negative and ten highest positive eigenvector scores in RDA. Rank of scores is listed along with frequency of records for each species in sites in the first and second surveys. Positive indicator species are identified by * and negative indicator species by #. Sources: JNCC (2004); Kirkham et al (2014).

	Rank of RDA score	Site records 1st survey	Site records 2nd survey	Change in site frequency
Species most associated with first survey (negative eigenvector scores)				
Poa trivialis	105	11	9	-2
#Bromus hordeaceus	104	9	4	-5
#Phleum pratense	103	9	4	-5
**Conopodium majus*	102	9	6	-3
Poa pratensis	101	4	1	-3
**Alchemilla glabra*	100	8	3	-5
Luzula campestris	99	6	2	-4
Alopecurus pratensis	98	8	6	-2
#Lolium perenne	97	10	9	-1
Cerastium fontanum	96	12	10	-2
Species most associated with second survey (positive eigenvector scores)				
#Ranunculus repens	1	5	14	+9
**Euphrasia spp*	2	5	11	+6
Plantago lanceolata	3	13	14	+1
**Scorzoneroides autumnalis*	4	5	10	+5
#Juncus articulatus	5	2	5	+3
Trifolium pratense	6	9	12	+3
Cynosurus cristatus	7	11	14	+3
#Holcus lanatus	8	13	14	+1
**Rhinanthus minor*	9	13	14	+1
Carex nigra	10	3	3	0

The analysis of homogeneity of community composition in the two sites revealed a significant decrease in the mean distances between centroids ($P = 0.04$). This result indicates that the vegetation has become more homogenous over the survey period. (Fig 7)

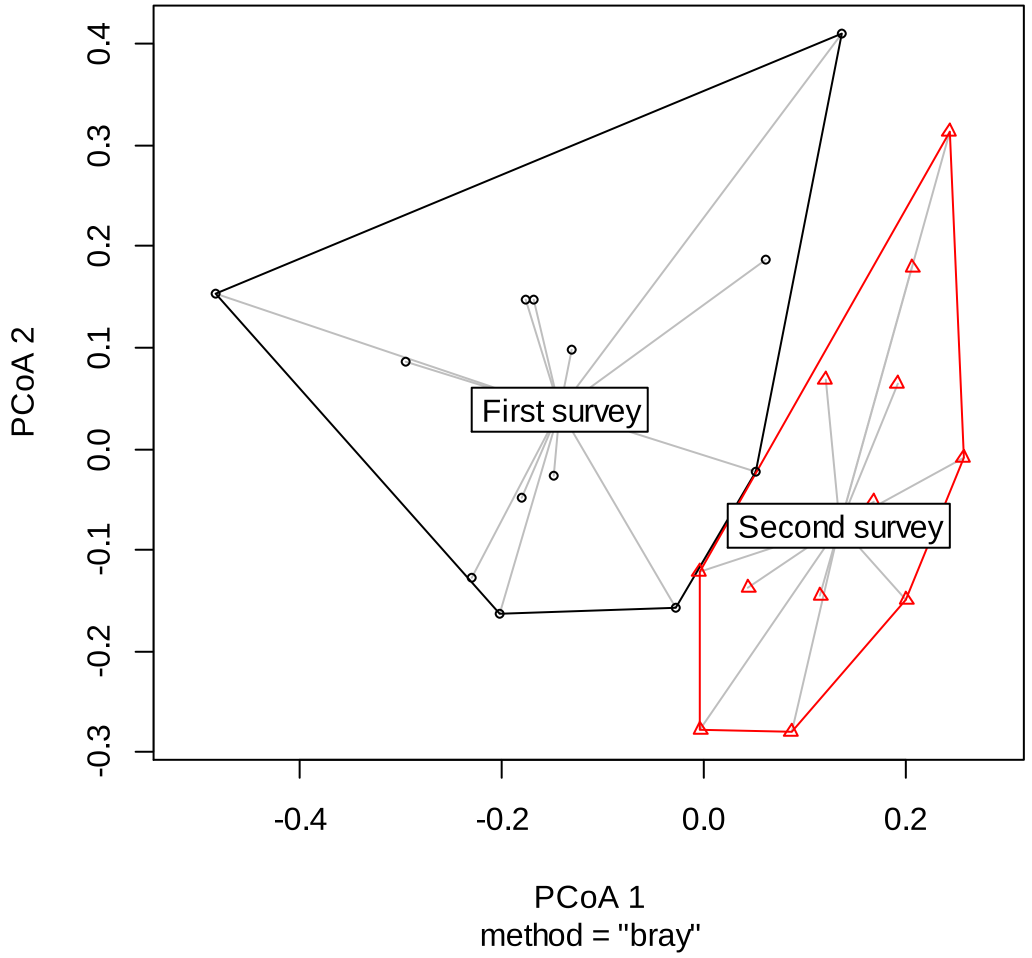

Fig. 7 PCoA plot showing analysis of homogeneity of multivariate dispersions with Bray Curtis distance matrix.

The analysis of percentage cover of positive meadow species in the first and second surveys did not reveal a significant difference but there was a significant decline in the percentage cover of negative species (see Table 4).

There was no significant difference in any of the mean Ellenberg Indicator Values (EIVs) between the two survey years (Table 4) suggesting that differences in soil conditions were minimal. However, when a comparison of the mean EIVs of the ten most influential species in each of the two surveys (listed in Table 3) was undertaken, there were significant differences (see Table 4). In this analysis EIV moisture increased, EIV reaction increased and EIV fertility decreased (Table 4).

Table 4 Comparison of percent cover of positive and negative meadow species; changes in Ellenberg indicator values (EIV): F (moisture), R (pH) and N (fertility) for all species and most influential species in RDA analysis. *P* values significant at the < 0.05 level are identified by*; *P* values of < 0.01 are identified by **; *P* values of < 0.001 are identified by ***. Non-significant results are identified by (NS).

	Test	**Result**	
		First survey	Second survey
Percent cover of positive species	Median	22.36	19.72
	Wilcoxon signed rank	W = 57, *P* = 0.807 (NS)	
Percent cover of negative species	Median	38.0	20.75
	Wilcoxon signed rank	W = 86, *P* = 0.036*	
Moisture EIV (all species)	Modified permutation test	F = 0.96, *P* = 0.307(NS)	
pH EIV (all species)	Modified permutation test	F = 3.05, *P* = 0.066(NS)	
Fertility EIV (all species)	Modified permutation test	F = 2.26, *P* = 0.111(NS)	
Moisture EIV (10 most influential species from each survey)	Median	5.29	5.55
	Wilcoxon signed rank	W = 26, *P* < 0.001***	
pH EIV (10 most influential species from each survey)	Median	5.95	6.13
	Wilcoxon signed rank	W = 52.5, *P* = 0.04*	
Fertility EIV (10 most influential species from each survey)	Median	5.59	4.38
	Wilcoxon signed rank	W = 187, *P* < 0.001***	

3.2 Genetic diversity and connectivity study

Table 5 shows mean and overall expected heterozygosity (H_e) and inbreeding coefficient (F_{IS}) values for the Bowland and Worcestershire populations. A full table of results is included in Appendix 4. Levels of inbreeding (F_{IS}) were relatively high ranging from 0.44 to 0.64. H_e in the Bowland populations was not significantly different from the values for the Worcestershire sites. Figure 3.5 shows that the results in Bowland and Worcestershire were similar to another UK study by Houston and Wolff (2012) but that levels of genetic diversity were considerably higher than those recorded in studies in Estonia (Talve and others 2013) and the Rocky Mountains (Hargreaves and others 2015).

Table 5 Results of genetic diversity and inbreeding analysis in Bowland and Worcestershire. Mean and overall values are shown.

Bowland	N	H_e	F_{IS}	Worcestershire	N	H_e	F_{IS}
Mean	28.2	0.387	0.577	Mean	31.2	0.340	0.544
Overall	254	0.472	0.648	Overall	281	0.434	0.583

N = number of samples; H_e = expected heterozygosity; F_{IS} = inbreeding coefficient.

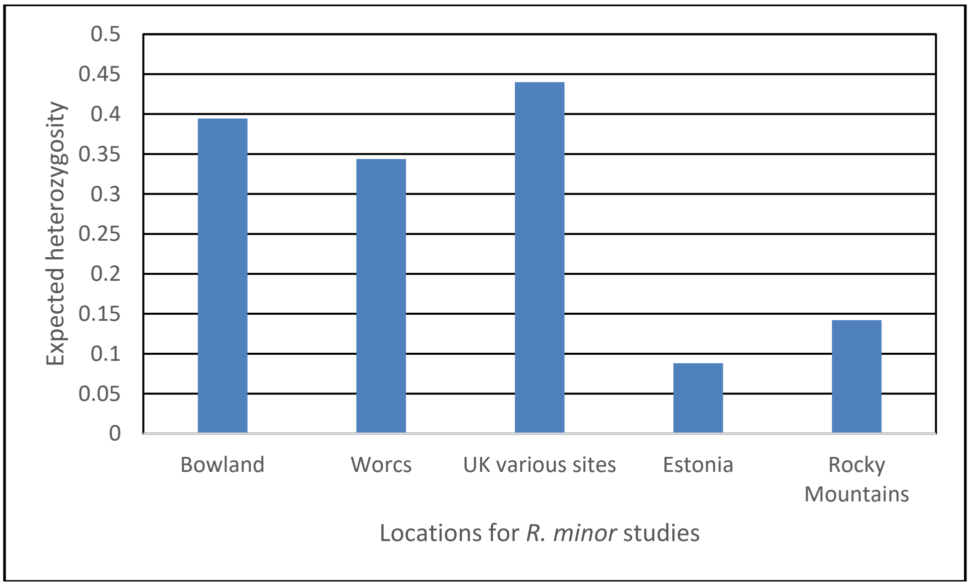

Fig. 8 Bar plot showing comparison of H_e results in Bowland and Worcestershire with those in other studies of *R. minor*

Population differentiation estimates using F_{ST} are shown in Table 6.

Table 6 F_{ST} values for meadow sites, and combination of meadow and intermediate sites in the two study regions. F_{ST} was lower in the Bowland region than Worcestershire which indicates greater population differentiation, and therefore less gene flow, in Worcestershire.

	F_{ST}
Bowland meadows	0.14
Bowland all sites	0.19
Worcestershire meadows	0.22
Worcestershire all sites	0.28

The spatial principal components analysis (sPCA) for both the Bowland and Worcestershire sites were in line with the F_{ST} results. Fig 9 is a colorplot of the first two components of the sPCA using the lagged scores from the analysis which reduce the ‘noise’ in the data. Similar colours in these plots indicate genetic similarity. Fig 10 shows a range of colours along the red green blue colour channel which indicates limited genetic similarity and, therefore, limited gene flow in this region. In the Bowland sites (Fig 9) intermediate sites and meadows in the south western cluster of sites are a similar colour indicating more gene flow between meadows and other sites. In Worcestershire there is no clear pattern indicating that there is limited gene flow between sites regardless of whether they are meadows or intermediate sites. However statistical analyses which form part of the sPCA confirmed that there was significant global structure in both regions which indicates limited gene flow, with a greater restriction on gene flow in the Worcestershire study area than in Bowland.

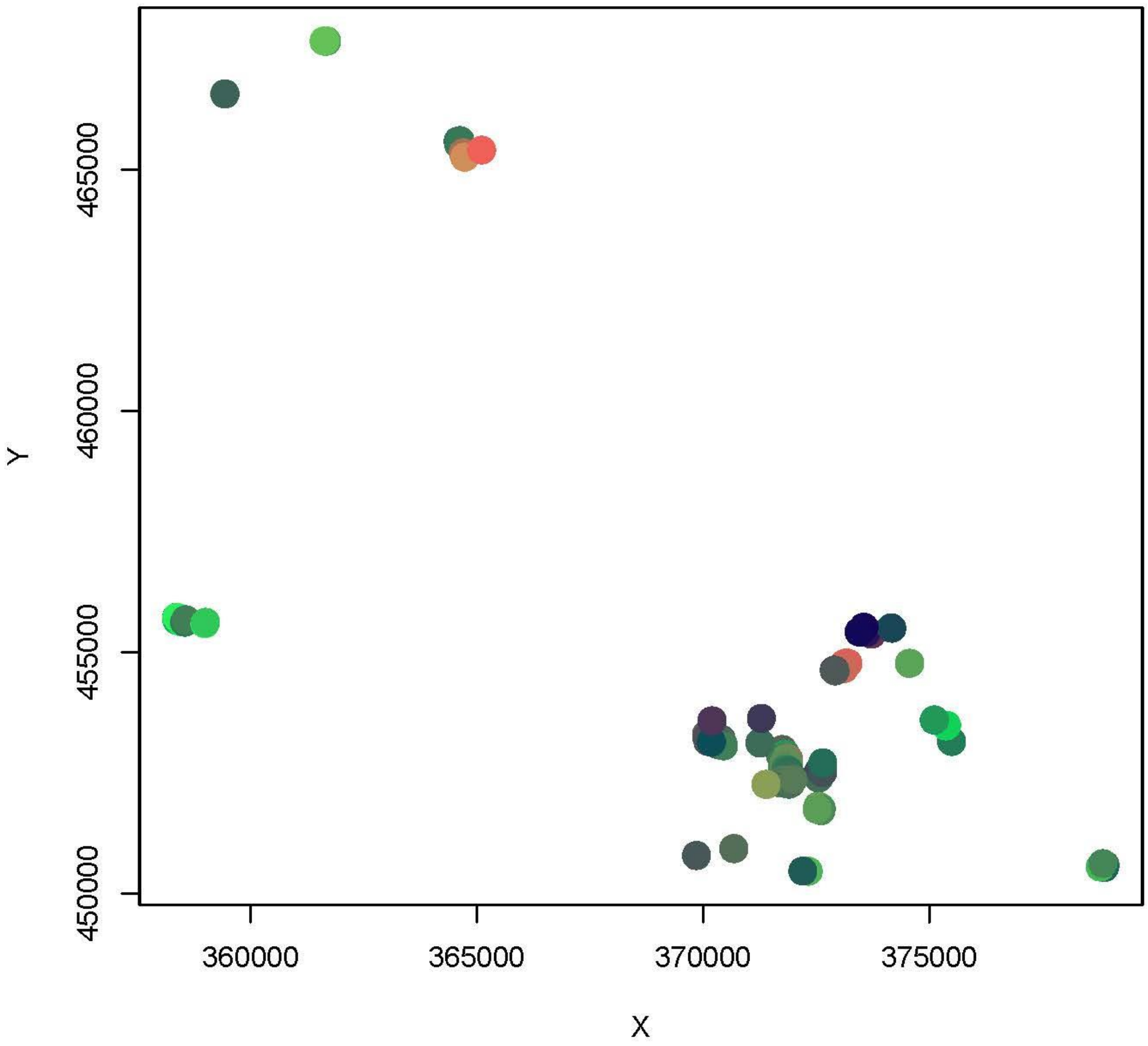

Fig. 9 Colorplot showing sPCA results for the Bowland meadows and intermediate sites. The more similar the colour, the higher the level of genetic similarity.

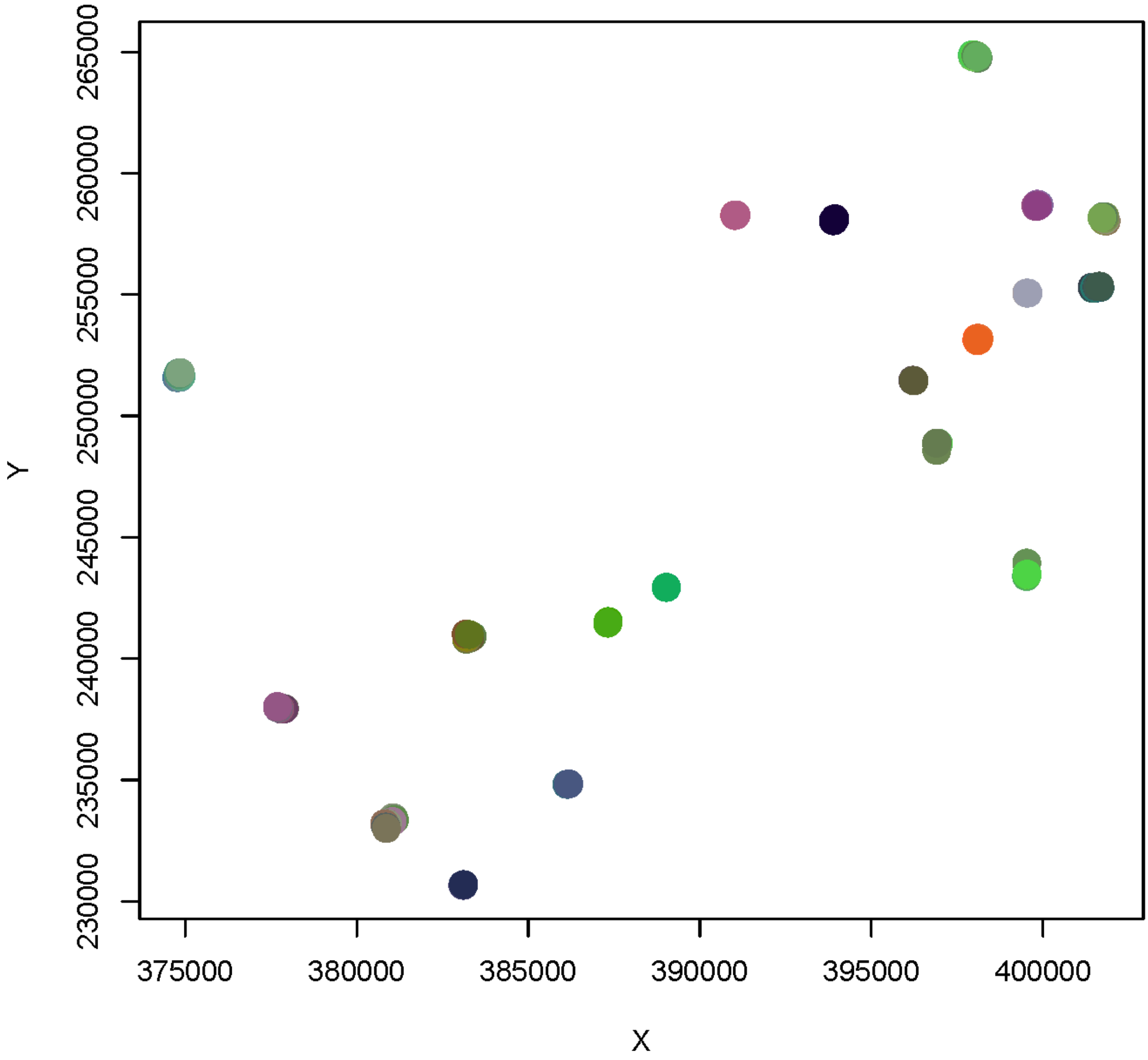

Fig. 10 Colorplot showing sPCA results for the Worcestershire meadows. . The more similar the colour, the higher the level of genetic similarity.

4. Discussion

Main discussion points:

- Grazed mesotrophic sites support diverse plant communities despite the lower number of designations and AES management agreements compared to meadows;
- Different survey methods both provided important data which were valuable in terms of monitoring change in a systematic quantitative way and by providing information about atypical parts of a site and picking up on rare species;
- A standardised approach to meadow management has been successful in maintaining diversity but this may also explain increasingly homogeneous meadow vegetation and potential loss of local distinctiveness;
- Moderate levels of genetic variation in meadow populations of *R. minor* indicate that meadow conservation is being successful in maintaining genetic diversity but gene flow (especially in Worcestershire) may be inhibited by more intensive land use.
- Grassland fragments outside of the meadows may play a role in an ecological network but more of these sites may be needed in areas of intensive land use.
- Further research on the effects of inbreeding (via self-pollination), other meadow species such as perennials and wind pollinated plants, and gene flow between 'old' and restored meadows would all add to the findings from this study.

4.1 Long-term change study

It was expected that there would be change in grasslands which had become more intensively managed because the impact of agricultural intensification on plant diversity is well documented (Hodgson and others 1999; Stoate and others 2009). However, change in community composition was also recorded in sites which had been consistently managed as meadows; and grazed mesotrophic sites were botanically diverse despite the fact that most were not protected sites, or part of a higher tier stewardship agreement. The impacts of the management of grazed, mesotrophic lowland grasslands sites have not been given as much attention as meadows in the literature (Stewart and Pullin 2008) but they can be very diverse and locally scarce species such as *Primula farinosa* and *Genista tinctoria* were recorded on these sites. On some grazed sites the botanical interest may have been maintained because the species rich areas were in places which were inaccessible to livestock. It may also be the case that change is slower in sites with a long history of extensive management (Cousins and Eriksson, 2002; Purschke *et al*., 2014).

The different results revealed by the use of both quadrat surveys and site species lists illustrated the value of a systematic, quantitative approach to measuring change in species richness and diversity, along with the advantages of a whole site survey which records change in atypical parts of the site. Species list surveys detected the presence of locally rare species such as *Cirsium heterophyllum* and *Platanthera chlorantha* which are also declining at the national level (Greenwood 2012).This combination of monitoring methods would identify gradual changes in plant populations, picking up on losses before they became critical, and would provide a more complete record of the diversity of species rich grassland sites. Current monitoring information could be compared with detailed baseline data, where it is available, such as survey data from the Lowland Grassland Inventory which was used in this study.

The detailed analysis of meadow sites showed that management had maintained the meadow plant community and led to a decrease in negative meadow indicator species, but it has not prevented turnover of positive indicator species, increased homogeneity of meadow vegetation and a net loss in the number of species recorded. Populations of plants can fluctuate from year to year due to environmental stochasticity which may be unrelated to longer-term trends (Shriver, 2016). However, some of the species which showed increases, such as *Ranunculus repens*, and decreases, such as *Alchemilla* spp., showed similar patterns of change over the same time period in another region of the UK (Starr-Keddle, 2014). It is possible that the standardised approach to meadow management, which is applied through AES prescriptions, may be maintaining a diverse meadow community at the regional level, but could also be suppressing local site distinctiveness to some extent. A study by Smith and others (2016) has suggested that spring and summer temperatures or biological indicators, such as the height or stage of development of key species, could be used to set dates for the removal of livestock in spring and for the hay cut, which could help to increase diversity and to tailor management to individual farms.

4.2 Genetic diversity and connectivity

The moderate levels of genetic diversity indicated that conservation of hay meadows was maintaining diversity in populations of *R. minor*, and suggested that fragmentation did not appear to be having a significant effect in larger populations of this key meadow species. However, levels of gene flow between sites were limited, particularly in Worcestershire where the landscape matrix between the sites with features such as major roads, rivers and areas of more intensively farmed land may present more of a barrier to pollinators in Worcestershire.

Findings from the analysis of *R. minor* populations in Bowland indicated that, whilst connectivity overall was limited, some intermediate sites which were in close proximity play a role gene flow. However, there was little evidence of connectivity between intermediate sites and meadows in Worcestershire. Fewer intermediates sites were found in Worcestershire and the ones that did support populations of *R. minor* were further away from the meadow sites (see Fig 3). It seems that there is a greater need for the restoration and enhancement of habitats which could function as part of the ecological network for meadow populations in an area like this where fragmentation appears to be having more of an impact.

It should be noted that this analysis only considered one species. It may be that there is less gene flow among populations of other meadow indicator species, and this is an important area for further research which could explain some of the losses of these species. Plants respond to fragmentation differently and genetic diversity varies according to different traits such as dispersal mechanisms and breeding systems even in plants within the same habitat (Thiel-Egenter and others 2009).

High levels of inbreeding were detected in both the Bowland and the Worcestershire populations of *R. minor* which can be explained by the tendency towards selfing and by poor seed dispersal in this species. The reasons for high levels of selfing and its consequences for the long term viability of populations are not fully understood. There is evidence for negative impacts of inbreeding on fitness but the extent of inbreeding depression and the varying effects on different species are still being debated (Angeloni *et* al., 2011; Angeloni *et al.*, 2014; Pico and van Groenendael, 2007). Whilst this uncertainty exists it would seem appropriate to take a precautionary approach to the conservation of species which exhibit high levels of inbreeding by ensuring that population sizes are as large as possible, and taking action to maximise pollinator abundance.

Grassland restoration has become a key part of the conservation strategy for the meadow habitat and is an option in the UK's Countryside Stewardship AES (Natural England, 2016). Assessment of the success of restoration has focused on species richness and diversity, and on functional traits in comparison with the donor site (Engst and others 2016; Kirkham and others 2012). Further research to investigate the potential for local adaptation by examining genetic similarity between donor and recipient site, and analyses of diversity and gene flow between donor and recipient sites over time would add a valuable measure of the long-term success of meadow restoration.

Implications for conservation

- This study has provided evidence that the conservation management of species rich meadows has been successful in maintaining a generally diverse meadow plant community and genetic diversity in populations of *Rhinanthus minor* although it may be possible to adjust the management regimes to enhance diversity and prevent further loss of some species.
- Systematic, quantitative and full species list monitoring would be valuable in assessing detailed long-term change and in identifying rare species and atypical parts of sites which add to their individual distinctiveness.
- Grazed mesotrophic sites are important in terms of botanical diversity and could be an additional priority for future designations and agri-environment management agreements.
- A more responsive approach to meadow management may prevent homogenisation of meadow vegetation and this could be based on spring temperatures or biological indicators, a recommendation which has been made in Smith (2016)'s study of upland hay meadows.
- Landscape-scale conservation of hay meadows should aim to maintain genetic diversity in key sites but would also benefit from action to manage grasslands and nectar sources outside of the meadow sites, especially in areas of more intensive land use.
- More research is needed to understand the increases in species such as *Ranunculus repens* and decreases in species such as *Alchemilla spp*.
- Further research would also help to answer questions about the reasons for high levels of inbreeding in *R. minor* and the possible links to pollinator abundance or the presence/absence of particular pollinator species.
- The genetic techniques used in this study could be used to investigate whether levels of diversity and connectivity are similar in other meadow species, and to assess gene flow between restored and 'old' meadow sites.

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Appendix 1 Meadow indicator species used in the long-term study

Positive and negative meadow indicator species in the Bowland meadow sites. Sources: Kirkham and others (2014); JNCC (2004).

Positive indicators	Negative indicators
Alchemilla glabra	*Bromus hordeaceus*
Alchemilla xanthochlora	*Cirsium arvense*
Caltha palustris	*Dactylis glomerata*
Centaurea nigra	*Heracleum sphondylium*
Conopodium majus	*Holcus lanatus*
Euphrasia spp	*Juncus spp*
Filipendula ulmaria	*Lolium perenne*
Geranium sylvaticum	*Phleum pratense*
Geum rivale	*Poa trivialis*
Lathyrus pratensis	*Ranunculus repens*
Leontodon hispidus	*Rumex acetosa*
Leucanthemum vulgare	*Rumex obtusifolius*
Lotus corniculatus	*Stellaria media*
Lotus pedunculatus	*Trifolium repens*
Persicaria bistorta	
Rhinanthus minor	
Sanguisorba officinalis	
Scorzoneroides autumnalis	
Stachys officinalis	
Succisa pratensis	

Appendix 2 Decreases and increases in numbers of site records for quadrat and species list data in the long-term study

Decreases in number of records of species at grassland sites for quadrat survey data and species list data. The 25 most decreased species are shown.

Quadrat survey data				**Species list survey data**			
Species	Site records (1st survey)	Site records (2nd survey)	Decrease in site frequency	Species	Site records (1st survey)	Site records (2nd survey)	Decrease in site frequency
Luzula campestris	20	6	-14	*Achillea ptarmica*	22	5	-17
Poa pratensis	14	2	-12	*Poa pratensis*	18	1	-17
Alchemilla glabra	15	4	-11	*Ficaria verna*	17	1	-16
Centaurea nigra	27	16	-11	*Luzula campestris*	25	12	-13
Achillea ptarmica	12	2	-10	*Achillea millefolium*	25	13	-12
Dactylis glomerata	20	10	-10	*Cardamine pratensis*	29	18	-11
Phleum pratense	17	7	-10	*Angelica sylvestris*	16	6	-10
Bellis perennis	18	9	-9	*Ajuga reptans*	13	4	-9
Bromus hordeaceus	13	4	-9	*Anemone nemorosa*	9	0	-9
Ficaria verna	12	3	-9	*Avenula pubescens*	14	5	-9
Conopodium majus	19	11	-8	*Alchemilla xanthochlora*	8	0	-8
Hypochaeris radicata	14	6	-8	*Cirsium vulgare*	8	0	-8
Leontodon hispidus	13	7	-6	*Festuca ovina*	16	8	-8
Plantago lanceolata	33	27	-6	*Leontodon hispidus*	19	11	-8
Prunella vulgaris	19	13	-6	*Ranunculus bulbosus*	9	1	-8
Trifolium repens	31	25	-6	*Bromus hordeaceus*	14	7	-7

Ajuga reptans	6	1	-5	*Centaurea nigra*	30	23	-7
Alchemilla xanthochlora	5	0	-5	*Conopodium majus*	24	17	-7
Anemone nemorosa	5	0	-5	*Phleum pratense*	21	14	-7
Juncus inflexus	6	1	-5	*Plantago major*	12	5	-7
Lathyrus pratensis	22	17	-5	*Vicia cracca*	21	14	-7
Ranunculus bulbosus	5	0	-5	*Agrostis capillaris*	34	28	-6
Sanguisorba officinalis	21	16	-5	*Cerastium glomeratum*	7	1	-6
Achillea millefolium	13	9	-4	*Heracleum sphondylium*	18	12	-6
Agrostis canina	4	0	-4	*Tussilago farfara*	8	2	-6

Increases in number of records of species at grassland sites for quadrat survey data and species list data. The 15 most increased species are shown.

Quadrat survey data				**Species list survey data**			
Species	No of site records (1st survey)	No of site records (2nd survey)	Gain in site frequency	Species	No of site records (1st survey)	No of site records (2nd survey)	Gain in site frequency
Ranunculus repens	20	31	11	*Alopecurus geniculatus*	8	19	11
Euphrasia species	8	13	5	*Galium palustre*	6	17	**11**
Galium palustre	3	7	4	*Juncus effusus*	15	25	10
Glyceria declinata	0	3	3	*Urtica dioica*	14	24	10
Lotus corniculatus	13	16	3	*Alopecurus pratensis*	14	23	9
Luzula multiflora	2	5	3	*Dactylorhiza fuchsii*	10	18	8
Myosotis discolor	5	8	3	*Juncus articulatus*	12	20	8

Alopecurus geniculatus	1	3	2	*Ranunculus repens*	28	35	7
Juncus effusus	5	7	2	*Myosotis discolor*	12	18	6
Trifolium dubium	1	3	2	*Poa trivialis*	26	32	6
Vicia cracca	8	10	2	*Euphrasia species*	13	17	4
Trifolium medium	0	1	1	*Galium aparine*	2	6	4
Triglochin palustre	0	1	1	*Glyceria declinata*	1	5	4
Urtica dioica	1	2	1	*Poa annua*	4	7	3
Vaccinium oxycoccos	20	31	11	*Alopecurus geniculatus*	15	18	3

Appendix 3 Abbreviations used in the redundancy analysis

Alchglab = *Alchemilla glabra*

Alopprat = *Alopecurus pratensis*

Bromhord = *Bromus hordeaceus*

Carenigr = *Carex nigra*

Cerafont = *Cerastium fontanum*

Conumaju = *Conopodium majus*

Cynocris = *Cynosurus cristatus*

Euphsp = *Euphrasia* species

Holclana = *Holcus lanatus*

Juncarti = *Juncus articulatus*

Lolipere = *Lolium perenne*

Luzucamp = *Luzula campestris*

Phleprat = *Phleum pratense*

Planlanc = *Plantago lanceolata*

Poaprat = *Poa pratensis*

Poatriv = *Poa trivialis*

Ranurepe = *Ranunculus repens*

Rhinmino = *Rhinanthus minor*

Scorautu = *Scorzoneroides autumnalis*

Triprat = *Trifolium pratense*

Appendix 4 Genetic diversity results

Bowland	***N***	***H_e***	***F_{IS}***	**Worcestershire**	***N***	***H_e***	***F_{IS}***
BG	21	0.450	0.582	BA	27	0.174	0.59
BS	88	0.507	0.620	BC	32	0.371	0.5
CB	12	0.408	0.460	EM	32	0.354	0.57
FH	20	0.034	0.665	HW	32	0.404	0.55
FHM	21	0.359	0.457	KP	32	0.391	0.55
LCM	18	0.339	0.604	LM	32	0.264	0.53
MM	40	0.441	0.591	SA	32	0.379	0.44
NI	12	0.390	0.677	SB	30	0.334	0.53
TB	22	0.463	0.565	SW	32	0.341	0.64
Mean	28.2	0.387	0.577	Mean	31.2	0.340	0.544
Overall	254	0.472	0.648	Overall	281	0.434	0.583

N = number of samples; H_e = expected heterozygosity; F_{IS} = inbreeding coefficient

Appendix 5 Pairwise F_{ST} results between the Bowland and Worcestershire meadows

Pairwise F_{ST} results for Bowland

	BG	BS	CB	FH	FHM	LCM	MM	NI
BS	0.02							
CB	0.05	0.02						
FH	0.09	0.03	0.19					
FHM	0.05	0.03	0.07	0.17				
LCM	0.09	0.03	0.13	0.17	0.13			
MM	0.03	0.03	0.04	0.08	0.03	0.06		
NI	0.04	0.01	0.02	0.22	0.08	0.11	0.04	
TB	0.07	0.02	0.03	0.14	0.10	0.11	0.08	0.04

Pairwise F_{ST} results for Worcestershire

	BA	BC	EM	HW	KP	LM	SA	SB
BC	0.14							
EM	0.17	0.09						
HW	0.12	0.09	0.03					
KP	0.18	0.11	0.06	0.05				
LM	0.30	0.13	0.12	0.12	0.11			
SA	0.13	0.04	0.02	0.03	0.05	0.13		
SB	0.17	0.09	0.11	0.06	0.09	0.10	0.08	
SW	0.16	0.14	0.13	0.06	0.07	0.17	0.10	0.06