Assessment of genetic diversity in populations of *Crepis foetida* L. (Asteraceae)

J. SQUIRRELL*, P. M. HOLLINGSWORTH

Royal Botanic Garden Edinburgh, 20A Inverleith Row, Edinburgh EH3 5LR

J. SEARS

Royal Society for the Protection of Birds, The Lodge, Sandy, Bedfordshire SG19 2DL

B. BANKS

English Nature, The Countryside Management Centre, Coldharbour Farm, Wye, Ashford, Kent TN25 5DB

B. FERRY

Royal Holloway, University of London, Egham, Surrey TW20 OEX

and

D. TH. DE GRAAF

RHC, Limburg, Sint Pieterstraat 7, NL 6211 JM Maastricht, The Netherlands

ABSTRACT

In 1980 *Crepis foetida* became extinct in Britain. Although several attempts have been made to re-introduce plants from native *ex-situ* collections into its formerly known locations, these attempts have largely been unsuccessful. We have used Amplified Fragment Length Polymorphisms (AFLPs) to assess levels of genetic diversity in these *ex-situ* populations by comparing them to *in-situ* European populations. Although the proportion of polymorphic loci in the British *ex-situ* populations was lower than in the French populations, gene diversity estimates in the British *ex-situ* and European *in-situ* populations were of a similar magnitude. British *ex-situ* plants were genetically more similar to Dutch plants than to those from French populations. There was no indication of a major bottleneck in the *ex-situ* populations or indication that limited genetic variation in these populations has been a major factor in the repeated failure of the re-introduction programmes.

KEYWORDS: AFLPs, *ex-situ*, extinction, re-introduction, Stinking Hawk's-beard.

INTRODUCTION

During 1980 the last remaining British population of *Crepis foetida* L. (Asteraceae), the Stinking Hawk's-beard, became extinct. *C. foetida* probably has never been common in Britain and is only known from a few coastal sites in south-east England (Preston *et al*. 2002). Globally, Britain represents the north-western limit of its range. Its distribution extends through Europe, where it is wide-spread, although it is more common in southern and eastern parts, to central Asia and the north-western Himalayas (Ferry 1999). Outside Britain it is not considered threatened, although populations are generally in decline (Anon 1998). It grows in open, well-drained sites in Britain, typically on disturbed shingle or chalk and reproduces sexually. The subspecies present in Britain and most of Europe (*C. foetida* subsp *foetida*) is considered to be self-compatible (Babcock 1947), unlike the two southern and eastern European subspecies

*Corresponding Author: j.squirrell@rbge.org.uk

C. foetida subsp. *commutata* and *C. foetida* subsp *rhoeadifolia*, which are self-incompatible (Babcock 1947; Hughes and Babcock 1950)

A recovery plan was initiated by the Nature Conservancy Council (now English Nature) in 1992, and *C. foetida* subsequently became a UK Biodiversity Action Plan (BAP) priority species in 1998, with the RSPB as Lead Partner. The Species Action Plan aimed to re-establish populations of *C. foetida* in former and new sites in south-east England. Plants cultivated at Cambridge Botanic Garden, originating from seeds collected from the last remaining plants at Dungeness, were used for the re-introduction programme. Funded in part by the RSPB and English Nature's species recovery programme, several attempts were made to re-establish populations at Dungeness and Rye during the 1990s and onwards (Sears 2004). Following visits to the Netherlands, where large populations exist in chalk quarries near Maastricht, populations were also established in three chalk quarries in Kent where the habitat reflected the conditions observed in the Netherlands. Although re-establishment appeared initially successful at Dungeness and Rye, the populations rapidly declined to a stage where there were no longer any plants remaining in the original re-establishment plots; a few new plants continue to be found each year at Rye. The populations in the Kent quarries were more recently introduced and their progress is still being monitored (Sears 2004).

The reason for the local extinction of *C. foetida* and apparent failure to survive re-introduction programmes remains unclear. It has been proposed that climatic and biotic factors have both played a role in its decline (Ferry 1999). On-going work at the Royal Holloway College is investigating which ecological conditions *C. foetida* requires for its survival. Seed set is good and long term seed viability remains high, to an extent that there is a good probability that a seed bank may exist at its former sites (Ferry 1999).

To contribute to this on-going work the aim of this paper has been to compare levels of genetic diversity in *ex-situ* British and *in-situ* European populations of *C. foetida*, to assess the extent to which genetic factors are likely to be contributing to the decline of UK populations of this species and to the difficulties with the reintroduction programmes.

MATERIALS AND METHODS

Plant material

During 2004, leaf samples of *C. foetida* were collected from 30 plants from the *ex-situ* population maintained in the Royal Holloway College. The plants from this collection were maintained in pots within a greenhouse and originated from seed originally collected from Dungeness. Leaves from 20 plants were also sampled from a second *ex-situ* collection, consisting of first generation self-sown plants, originating from three pot grown plants from the Dungeness/Royal Holloway College collection and maintained in a private garden.

To compare levels of genetic diversity, two French populations were sampled south of Clermont-Ferrand, Auvergne, a region where this species is widespread. The Saurier population was found in a small cutting at the foot of a cliff beside a road, while the plants from the St. Floret population were growing on bare ground beside a track. 25 plants were sampled from both populations. Samples were also obtained from the Netherlands. Two populations were sampled from quarries (ENCI and 't Rooth, 25 and 20 plants sampled respectively) near Maastricht, and five plants from an *ex-situ* population in the garden of the Maastricht Natural History Museum which originated from plants from the ENCI quarry population.

DNA extraction and AFLP analysis

DNA was extracted from approximately 25 mg of leaf material that had been previously dried in silica gel, using a miniprep version of the 2xCTAB (cetyltrimethyl-ammonium bromide) method of Doyle & Doyle (1987). Genetic diversity was assessed using Amplified Fragment Length Polymorphisms (AFLP; Vos *et al*. 1995). AFLP analysis was performed using method adaptors and pre-amplification primers described in Woodhead *et al*. (2005). The resulting fragments were separated using a CEQ 800 system (Beckman Coulter) and subsequent output files were analysed using the associated fragment analysis software (Beckman Coulter). Twelve selective primer pair combinations were initially screened for suitable fragment amplification, scorability and reproducibility, using four individuals each from France and Britain.

Of these, three primer pairs were selected for large scale screening of individuals:

E-AGC (5'GACTGCGTACCGGTTCAGC) with
M-CTT (5'GATGAGTCCTGCGTAACTT)

E-AAC (5'GACTGCGTACCGGTTCAAC) with
M-CTA (5'GATGAGTCCTGCGTAACTA)

E-ACC (5'GACTGCGTACCGGTTCACC) with
M-CAA (5'GATGAGTCCTGCGTAACAA).

The primers E-AGC and E-AAC were labeled with the fluorescent dye D4-PA, and the primer E-ACC with the dye D3-PA (WellRED oligos, Proligo). Bands were scored as either present (1), absent (0) or ambiguous (?) to generate a binary matrix.

Data analysis

A Bayesian approach was used to estimate allele frequencies using the program AFLP-SURV (Zhivotovsky 1999). The percentage of polymorphic loci (5% level) was calculated and unbiased estimates of gene diversity (*H*j) were made following the method of Lynch and Milligan (1994) at the level of the population, country and for the species. Estimates of population differentiation (F_{ST}) were also calculated, and significance tested, using AFLP-SURV. Multilocus similarities between individuals were assessed using Jaccard's similarity co-efficient and subjected to principle co-ordinate (PCO) analysis using R-Package version 4.0 (available from http://www.bio.umontreal.ca/casgrain/en/labo/R/v4/index.html) and visualised graphically using Excel.

RESULTS

79 individuals were successfully genotyped using the three primer pair combinations. Only six individuals of the 130 samples collected from the three Netherlands populations in total were successfully genotyped, in spite of repeated DNA extraction and AFLP analysis. For AFLPs to work successfully, large amounts of high quality DNA are required, so the continual failure of some samples suggests that the initial leaf material collected was of non-optimal quality. As a result, the six individuals were grouped together into a single population representing the Netherlands.

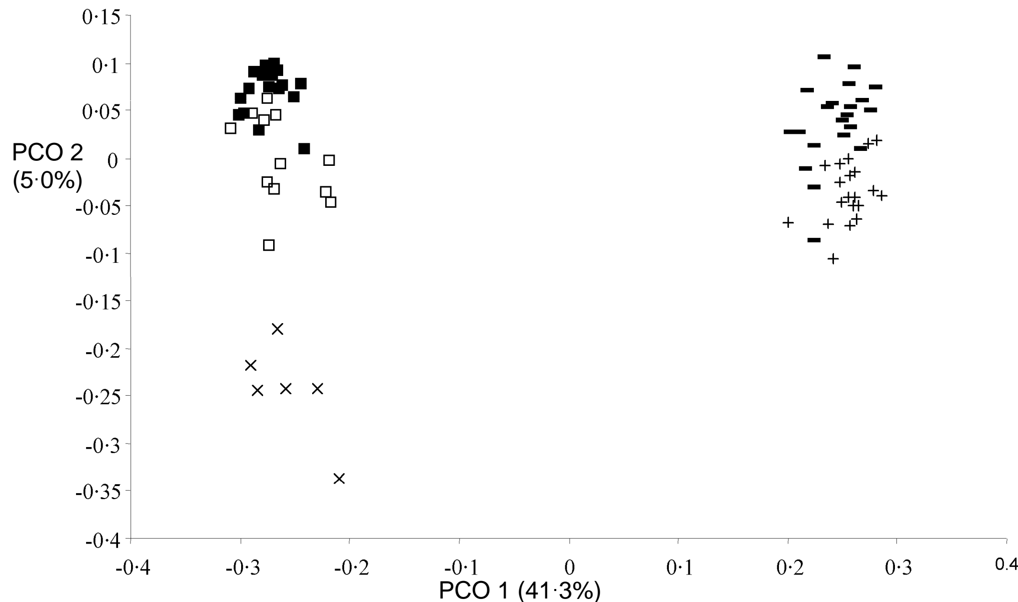
In total 298 bands were scored from the three primer pair combinations (E-AGC + M-CTT 102 bands between 60 and 300 bp; E-AAC+M-CTA 91 bands between 55 and 205 bp; E-ACC+M-CAA 105 bands between 80 and 286 bp) of which 93·3% (95% level) were polymorphic.

The percentage of polymorphic loci ranged from 46·6% in the private garden to 62·4% in the St. Floret population (Table 1). At the country level, France had more polymorphic loci compared to the British populations (63·1% vs 46·6% respectively). Gene diversity estimates ranged from 0·139 (private garden) to 0·204 (St. Floret) and at the country level was higher in France compared to Britain (0·196 vs 0·173 respectively). Significant levels of population differentiation were observed between British and French populations, and at the global level (Table 1).

TABLE 1. GENETIC DIVERSITY PARAMETERS OF *IN-SITU* AND *EX-SITU* POPULATIONS OF *CREPIS FOETIDA* BASED ON 298 AFLP LOCI, AT THE POPULATION, COUNTRY AND GLOBAL LEVEL

Population	n	*P*	*H*j	F_{ST}
Britain *ex-situ*				
Private garden	20	46·6	0·139	
Royal Holloway	12	47·7	0·201	
mean	15·5	47·2	0·170	
France *in-situ*				
Saurier, Auvergne	20	57·7	0·185	
St. Floret, Auvergne	21	62·4	0·204	
mean	20·5	60·5	0·195	
Netherlands				
Maastricht	6	48·8	0·155	
Countries				
Britain	32	46·6	0·173	0·106*
France	41	63·1	0·196	0·066*
Netherlands	6	48·8	0·155	-
mean	26·3	52·8	0·175	0·086
Global overall	79	68·1	0·235	0·336*

n, number of individuals genotyped; *P*, percentage polymorphic loci; *H*j, expected gene diversity; F_{ST}, population differentiation; *, $p < 0·01$.



Britain: □ Royal Holloway College, ■ Private Garden. France: ▬ Saurier, ✚ St. Floret. × The Netherlands.

FIGURE 1. Principal co-ordinates plot based on the similarity matrix between individuals from both *in-situ* and *ex-situ* populations of *Crepis foetida*, calculated using 298 AFLP bands. Axes explain 46·3% of the variation.

Results of the principal co-ordinates analysis are presented in Fig. 1. Combined axes *x* and *y* explained 46·3% of the variation. The *x* axis clearly distinguished the French populations from the British and Dutch samples and, to a lesser degree, the *y* axis distinguished the British from the Dutch samples. Within France there was some overlap between populations, while the private garden population formed a tight cluster with the individuals from the Royal Holloway population (Fig. 1).

DISCUSSION

The main outcome of this research, although based on only a limited number of plants, is that the *ex-situ* plants of *C. foetida* maintained at the Royal Holloway College still harbour genetic variation and that extensive loss of genetic diversity has not occurred. Although the proportion of polymorphic loci was about 17% lower than in French populations with equivalent sampling, there was no major difference in genetic diversity as assessed by *H*j. Taken together, these results suggest that the loss of genetic diversity in the Royal Holloway population compared to the French populations is at present limited.

Previous studies have shown that loss of genetic diversity can be directly linked to population extinctions (Sacherri *et al.* 1998) and that populations at the edges of distribution ranges tend to exhibit less genetic diversity compared to populations in the centres of their ranges. However, populations that are isolated or are at the edge of ranges can exhibit levels of genetic diversity that are higher than expected. For example, two small isolated range edge populations of *Lloydia serotina* in Wales had unexpectedly high levels of genetic diversity (Jones *et al.* 2001). The Welsh populations showed diversity levels similar to European Alp populations and even exhibited higher levels than some North American populations. Our data suggest that prior to the extinction of *C. foetida* in 1980 comparable levels of genetic variation to European populations were present in the UK and that the majority of this variation has been maintained in the *ex-situ* collection.

In contrast to the Royal Holloway population, there is more limited variability in the second *ex-situ* population from the private garden. This lack of genetic variation was not unexpected and resulted from the fact that they originated from seed from just three plants grown at the Royal Holloway College. Ideally,

re-introductions should be made up from plants containing high levels of genetic diversity to maximise the chances of including plants containing genes suitable for the survival and adaptation of the population, as well as avoiding inbreeding depression. Re-introductions using the plants solely from the private garden population should therefore be avoided.

Ideally, re-introductions should be made from donor populations that match as closely as possible the ecological and genetic make-up of the original population. Given the lack of evidence for an extreme genetic bottleneck in the UK *ex-situ* material, there appears no clear-cut and strong reason to introduce Continental material to the former sites in the UK. It would, however, be interesting to establish new sites based on continental material, to compare its performance with the UK material. Establishing new sites, rather than supplementing the existing site would remove concerns about harming the UK population via outbreeding depression either by (a) introducing genes not adapted to the local environment, or (b) disrupting any co-adapted gene complexes.

Our results suggest that the Dutch plants are more similar genetically to the British *ex-situ* population than the French population, albeit with the qualifier that only a limited number of populations have been examined. Specimens from the Auvergne region are reported to be the same subspecies as that formerly present in Britain (Babcock 1947) and it is considered that the dissimilarity between populations is indicative of their geographical isolation rather than taxonomic differences between the UK and European populations.

CONCLUSION

In the absence of comparative tests of fitness and adaptation via reciprocal transplant and common garden experiments our results need interpreting with some caution. However, what can be said is that no evidence for a severe genetic bottleneck has been detected in the Royal Holloway population. A tentative interpretation of this is that levels of genetic variation in British *C. foetida* have not been the main contributing factor that has led to its extinction and continual re-introduction failure, and that these plants represent a potentially useful source for future re-introduction of the native genotypes. Instead a combination of ecological factors, such as habitat change and climatic conditions, may potentially have played a greater role in the decline of this species in Britain than have genetic factors.

ACKNOWLEDGMENTS

We wish to thank Michelle Hollingsworth and Alex Clark for laboratory assistance and the RSPB for providing funding support. The Royal Botanic Garden Edinburgh is supported by the Scottish Executive Environment and Rural Affairs Department.

REFERENCES

ANON. (1998) *UK Biodiversity Group: Tranche 2 Action Plans. Volume 2*. pp 87–88. English Nature, Peterborough.

BABCOCK, E. B. (1947). *The genus* Crepis. University of California Press, Berkeley and Los Angeles.

DOYLE, J. J. & DOYLE, J. L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf material. *Phytochemical Bulletin* **19**: 11–15.

FERRY, B. W. (1999). *Crepis foetida* L. (Asteraceae), in M. J. WIGGINGTON ed. *British Red Data Books*, pp 108. JNCC, Peterborough.

FRANKHAM, R. (2003) Genetics and conservation biology. *Comptes Rendus Biologiques* **326**: S22–S29.

HUGHES, M. B. & BABCOCK, E. B. (1950). Self-incompatibility in *Crepis foetida* (L.) subsp. *rhoeadifolia* (Bieb.) Schinz et Keller. *Genetics* **35**: 570–588.

JONES, B., GLIDDON, C. & GOOD, J. E. G. (2001). The conservation of variation in geographically peripheral populations: *Lloydia serotina* (Liliaceae) in Britain. *Biological Conservation* **101**: 147–156.

PRESTON, C. D., PEARMAN, D. A. & DINES, T. D. (2002). *New Atlas of the British and Irish flora*. Oxford University Press, Oxford.

SACCHERI I., KUUSSAARI, M., KANKARE, M., VIKMAN, P. *et al*. (1998). Inbreeding and extinction in a butterfly metapopulation. *Nature* **392**: 491–949.

SEARS, J. (2004). *The Stinking Hawk's-beard*. http://www.rspb.org.uk/biodiversity/RSPBandbiodiversity/lead_partner/plants/hawksbeard/index.asp (as of April 2006).

WOODHEAD, M., RUSSELL, J., SQUIRRELL, J., HOLLINGSWORTH, P. M. *et al*. (2005). Comparative analysis of population genetic structure in *Athyrium distentifolium* (Pteridophyta) using AFLPs and SSRs from anonymous and transcribed gene regions. *Molecular Ecology* **14**: 1681–1695.

VOS, P., HOGERS, R., BLEEKER, M., REIJANS, M. *et al*. (1995). AFLP: a new concept for DNA fingerprinting. *Nucleic Acids Research* **23**: 4407–4414.

ZHIVOTOVSKY, L. A. (1999). Estimating population structure in diploids with multilocus dominant DNA markers. *Molecular Ecology* **8**: 907–913.

(*Accepted April 2006*)