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Genetic aspects of the Species Recovery Programme for the Plymouth pear *Pyrus cordata* Desv.

Andy Jackson, Berenice Erry and Alastair Culham

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Genetic factors have been pivotal to the Species Recovery Programme for *Pyrus cordata*. The primary target was to safeguard the two existing populations by ensuring long-term viability. Using the molecular technique of Randomly Amplified Polymorphic DNA (RAPD) only two clones have been identified, one from each of the two reproductively isolated populations in south-west Britain. This low level of genetic diversity was illustrated by the results of controlled pollinations within and between these populations. Self- and cross-incompatibility was exhibited within each of the populations, where individuals were separated by up to 1.9 km. However, when crossed, the two reproductively isolated populations were compatible. With only two clones, minimum allele frequencies at any locus in the British populations are 0.25. This means that a maximum of four or a minimum of three *S* alleles are controlling self-incompatibility. The loss of a single *S* allele could have a significant effect upon future reproductive performance and therefore the medium-term conservation of the British representatives of *P. cordata*. To conserve the remaining genetic diversity and thereby ensure long-term viability, an experimental population was designed to bring together the two genetically distinct and currently reproductively isolated clones. The first phase of planting commenced in 1995. In the longer-term, it is intended to review this strategy following a study of the genetic diversity of *P. cordata* throughout its distribution in Europe.

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Introduction

The Plymouth pear *Pyrus cordata* Desv. is one of Britain's rarest wild trees. It is the only tree species to be protected under Schedule 8 of the Wildlife and Countryside Act 1981. In 1991 it became one of the first six taxa in the Species Recovery Programme managed and funded by English Nature. Its selection was justified on the basis of its rarity in Britain, its statutory protection and concern for the security of the wild populations in the long-term. As a wild relative of the cultivated pear *P. communis*, with which it can freely hybridise, it is also worthy of conservation interest as a potential genetic resource for future breeding programmes.

The overall strategy of the recovery programme for *P. cordata* combines *in-situ* and *ex-situ* initiatives which are founded on an understanding of the complex, interacting disciplines of genetics, reproductive biology, ecology and horticulture (Jackson 1995). Genetic factors have been pivotal to the recovery programme and their detailed consideration was essential for the design of a medium-term conservation strategy.

The distribution of *Pyrus cordata*

Pyrus cordata is naturally restricted to south-western Europe with populations in France, particularly Brittany, and the north-west of Spain and Portugal. The two populations known in south-west Britain are its most northerly locations and, although geographically disjunct, are linked to mainland populations by similarities in climate, flora, geology and history of human disturbance. Human occupation of this region has resulted in a predominantly agricultural landscape interspersed with larger blocks of semi-natural and planted woodland which are interconnected by hedgerows and riverine woodland.

The British populations

Anthropogenic habitats dominate the British populations. In Plymouth, Devon, the once rural hedgebank where it was first discovered in 1865 (Archer Briggs 1880) is now subsumed within a light-industrial estate. Despite local protestations, a significant proportion of the original hedgebank was destroyed during development in the 1970s. In 1989 a second hedgebank was found 1.6 km to the east and today a total of 20 individual stems have been uniquely numbered from within these hedgebanks and the several individuals which were transplanted in the 1970s. Numerous small suckers occur within the dense hedgerows and 28 of these have been transplanted to semi-natural areas managed as Local Nature Reserves by Plymouth City Council.

In Cornwall five sites have been located near Truro, separated by up to 1.9 km. The first of these was confirmed from voucher specimens collected in 1989 and the last was found as a result of publicity in the local press in 1991. Over 80 individual stems ranging from 1 cm to 46 cm diameter have been individually tagged and 450 other small plants have been recorded and mapped (Tonkin 1993).

Owing to the ability of *P. cordata* to produce an abundance of suckers it is often difficult in the field to identify what should be regarded as an individual plant worthy of a unique number. Furthermore, segregating the population into sexual and asexual propagules is totally impractical. Attributing an age to individuals, as estimated by stem diameter and height, must also be considered in the light of this asexual reproduction and it is likely that the individuals are much older than their measurements might indicate because of the impact of management events such as hedge-cutting.

Threats to the British populations

All but one of the British sites of *P. cordata* are protected within Sites of Special Scientific Interest (SSSIs). However, unintentional damage can still occur through agricultural practices such as hedge-cutting, grazing or pesticide drift. The provisions of the Wildlife and Countryside Act should reduce the likelihood of the wholesale habitat destruction that occurred to the Plymouth population in the 1970s. The theft of the entire fruit production from controlled cross-pollinations in 1994, however, is an indication of one of the more subtle human pressures. Pathogens such as fireblight *Erwinia amylovora* have the potential to eradicate entire populations quickly and must be constantly monitored.

Most of these factors are of short-term importance. If *P. cordata* is to become self-sustaining in the long-term, then the greatest concern must centre around its ability to adapt to changes in the local environment. This will be dependent largely on the genetic variation of individuals within populations arising through propagation by seed. Therefore, reproductive isolation, leading to low seed-set, and inbreeding depression where incompatibility occasionally breaks down, are both immediate and long-term threats to the genetic heterogeneity of the populations.

The need to identify genetic variation

Considerable morphological difference was identified between the Plymouth and Truro populations. This included leaf shape, flowering time, carpel number and the overall form of the trees. On morphological evidence alone the two populations were thought to be genetically distinct. Indeed, initial concern focused on whether both populations were the same taxon. This was resolved only after a thorough literature review and on comparison with herbarium specimens from mainland Europe. Variation within each population was restricted to flowering time and growth habit. Much of this was apportioned to differences in the local microclimatic and edaphic conditions.

An early indication of a low level of genetic variation within the British populations was gained from the results of both open and controlled pollinations. Before the Recovery Programme began the annual fruit production of the Plymouth population had been observed to be sporadic. Records of viable seeds existed but no detailed accounts were available. Then, in 1992, an unusually large harvest of 1178 open-pollinated fruits yielded only 19 fully developed seeds. Germination tests on Plymouth seeds indicate 80% viability (Jackson 1993), but the seedlings are not vigorous. A study of pollen tube growth confirmed that self-incompatibility, by prevention of normal pollen tube development, prevented fertilisation within the Plymouth population. Controlled pollinations confirmed this incompatibility with 172 self-pollinations and 141 pollinations between individuals producing no viable seeds.

This result indicated the need to employ a DNA fingerprinting technique to identify genetically distinct individuals in order to direct a hybridisation programme.

It was necessary to study genetic diversity at three primary levels:

- among individual plants;
- among sites within the populations;
- between the two main populations (Truro and Plymouth).

In addition, hybridisation experiments demonstrated that viable seed could be readily obtained by crossing *P. cordata* with a wide taxonomic range of wild pears and with cultivated varieties of *P. communis* such as 'Williams' and 'Conference'. Given that some seed had been obtained through open-pollination in the Plymouth population there was further concern about the possibility of introgression and loss of distinctiveness between the wild populations of *P. cordata* and cultivated pears nearby. The final question was, therefore, whether true *P. cordata* could be identified at the species level using genetic markers.

DNA fingerprinting

The complex distribution of the British populations and the difficulty of identifying unique individuals in the dense hedgerows presented practical problems in deciding which plants to sample and how to identify them. These decisions were essential precursors to the DNA analysis given that the aim was to return to the plants to conduct a cross-pollination programme. Over 120 of the largest individual stems were uniquely numbered and more than 460 other small plants were recorded and mapped. The 120 numbered plants were used for sampling. Fresh leaves were gathered from each and duplicate DNA extractions made for each numbered individual.

There are several plant genetic fingerprinting techniques available, each requiring a slightly different knowledge of the organism under study and each with particular limitations in terms of time, cost and quantity of DNA required. The smallest plants had only ten leaves and availability of DNA was

therefore one of the primary limiting factors.

One of the most widely used fingerprinting techniques, Randomly Amplified Polymorphic DNA (RAPD) (Welsh & McClelland 1990; Williams *et al.* 1990) was considered particularly applicable to this project for the following reasons.

- The quantity of leaf material, and therefore quantity of DNA available, was very small for many individuals.
- Sampling needed to be as non-destructive as possible. With certain techniques this requirement would have limited the sample size so severely that the between-individual genetic variation could not have been investigated.
- The number of individuals to be screened was large, and both time and resources were limited.
- The plants under study are long-lived woody perennials and particular banding patterns could, therefore, be associated with particular plants for the full duration of this project, and throughout the ensuing Recovery Programme.

The use of the M13 probe (Ryskov *et al.* 1988) for DNA fingerprinting was evaluated, but too many bands were generated for easy comparison of individuals. Also, the relatively large amount of DNA required per individual for one trial (11 μg) was far more than could be extracted from small plants if the patterns were to be confirmed by repetition of the experiment.

RAPDs require little DNA because the procedure amplifies the target DNA; it requires no prior knowledge of the genome because the primers are random; and the technique can be applied to large sample sizes very quickly (see Berry, Crawford & Hewitt (1992) for an overview of techniques and applications). The resolution of the technique is somewhat governed by the number of primers that can be screened and, in most cases, banding patterns show dominance so heterozygotes cannot be distinguished. The variation in banding patterns is therefore largely that of presence or absence rather than band shifting.

Three sets of primers of different origin were used: Williams *et al.* (1990) – 10 primers; Operon Technologies Inc. (Set E) – 20 primers; University of British Columbia (UBC) (Set 8) – 100 primers. This final set was chosen as a result of reports indicating that they were more sensitive at detecting genetic polymorphism.

Samples were taken from 123 numbered plants ranging from mature trees to stems less than 20 cm tall. DNA was extracted from either fresh leaves or leaves dried by silica gel, using the CTAB extraction method (Doyle & Doyle 1987). DNA quality and quantity were assessed prior to dilution suitable for the polymerase chain reaction (PCR).

The first 75 DNA extractions, representing both of the main Plymouth sites and all five Truro sites, were screened with the Williams primer set. Two plants from each of these seven sites were screened with Operon primer Set E. The UBC primer set was too large for such comprehensive initial screening to be undertaken and, therefore, the strategy adopted was to take two plants each from Truro and Plymouth, which represented a total of four sites chosen on the basis of widest geographical separation. Primers which appeared, on first examination, to show polymorphism were then used to screen individuals taken from all sites. Three varieties of *P. communis* were surveyed alongside *P. cordata* using a subset of the primers.

There was no reproducible variability within *P. cordata* at any of the three levels of study for 98% (126) of the primers (Figure 1). The remaining four

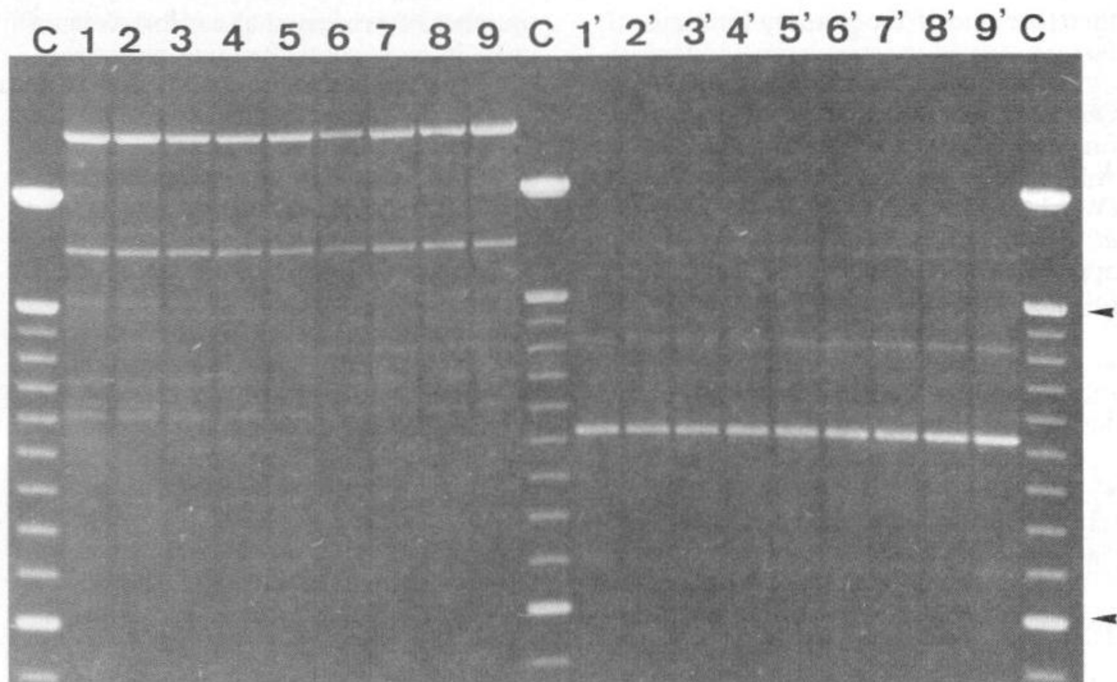

Figure 1. Sample RAPD primers showing no band polymorphism between Truro and Plymouth populations of *Pyrus cordata*.

Lanes 1–5 and 1'–5' show Truro plants from sites 1–5 respectively. Lanes 6–9 and 6'–9' show Plymouth plants from Marjon, Plymbridge, Tecalemit and Wrigleys, respectively. Lanes 1–9 show primer UBC705 and lanes 1'–9' show primer UBC710. Lanes marked 'C' contain the Gibco 100 bp DNA weight marker (600 bp and 1500 bp markers are indicated with an arrow).

(numbers 717, 756, 779, 785), all from the UBC set, detected differences, but only between the two main populations. Primer 717 showed bands at 500, 570, 660, 1000 and 1190 bp for both Plymouth and Truro populations but had a unique band at 420 bp in Truro populations and bands at 460 and 890 bp in Plymouth populations. Primer 756 showed bands unique to Plymouth trees at 700 (faint), 1650 and 1800 bp and bands unique to Truro trees at 300 and 1000 bp (Figure 2). Primer 779 showed common bands at 950 and 1900 bp, bands unique to Truro populations at 1000, 1400 and 1500 bp and bands unique to Plymouth populations at 1020, 1150 and 1550 bp. Primer 785 showed bands at 520, 780, 910, 1100, 1440 and 1500 bp with one band unique to Plymouth populations at 710 bp.

These primers were then used to compare *P. cordata* with cultivated *P. communis*. They successfully distinguished between them and ruled out the possibility of introgression of these two species in the current British populations (Figure 3).

Utilisation of the genetic data

The morphological and RAPD variation between the populations was confirmed in a crossing programme. In the first year (1994), 258 crosses between the Plymouth and Truro populations yielded 43 fruits containing 93 seeds of which 72% germinated. The British populations of *P. cordata* are, therefore, thought to consist of only two reproductively isolated clones. In order to conserve these clones and to endeavour to secure at least medium-term viability, an experimental population was designed.

In conservation genetics much debate

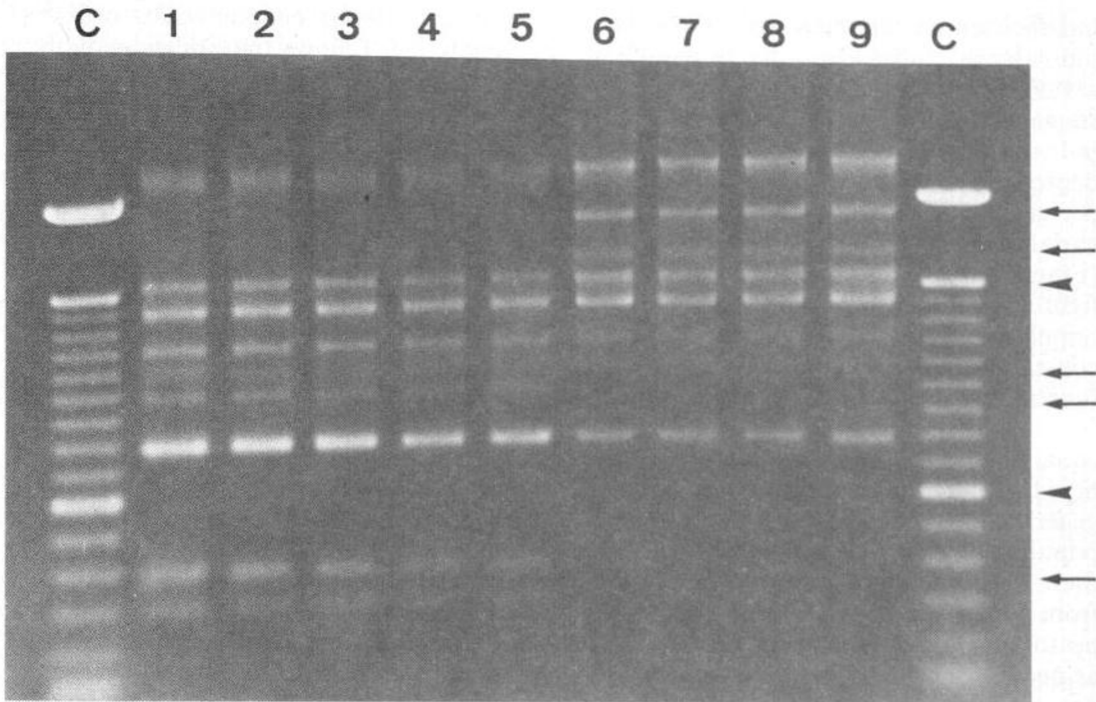

Figure 2. RAPD primer UBC756 shows band polymorphism between Truro and Plymouth populations.

Lanes 1–5 are samples from Truro sites 1–5, respectively. Lanes 6–9 are from Plymouth sites Marjon, Plymbridge, Tecalemit and Wrigleys, respectively. Lanes marked 'C' contain the Gibco 100 bp DNA weight marker (600 bp and 1500 bp markers are indicated with a short arrow ▷). Polymorphisms are indicated with a long arrow →.

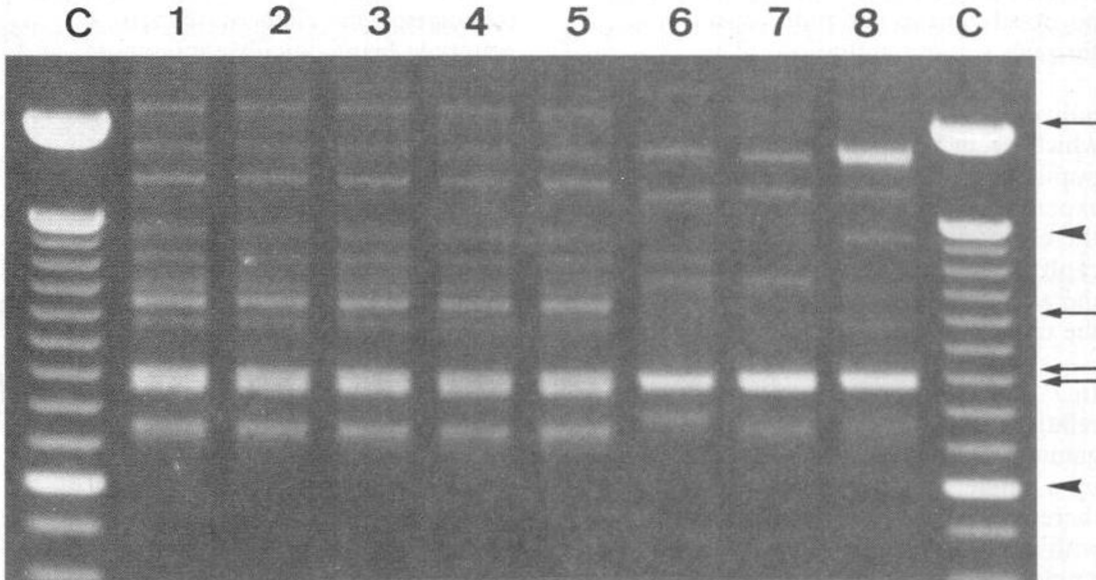

Figure 3. RAPD primer UBC724 illustrates differences found between *Pyrus cordata* and *P. communis*.

Lanes 1–3 are samples from Truro sites 1, 2 and 4, respectively. Lanes 4 and 5 are from Plymouth sites Marjon and Plymbridge, respectively. Lanes 6–8 are *P. communis* cvs Doyenne de Comice, Williams and Conference, respectively. Lanes marked 'C' contain the Gibco 100 bp DNA weight marker (600 bp and 1500 bp markers are indicated with a short arrow ▷). Polymorphisms are indicated with a long arrow →.

has focused on the minimum number of individuals required in order to maintain a viable population. It has been suggested that for most species, 50 individuals could reduce inbreeding depression to an acceptable level of 1% per generation and 500 could prevent variation being lost through genetic drift (Franklin 1980; Soulé 1980). For these figures to be relevant, the breeding population size needs to remain constant; the numbers of individuals functioning as males and females should be equal; all individuals should have an equal chance of producing offspring that breed in the next generation; and the generations should not overlap. For populations reintroduced into the wild these are difficult to control and figures from 5000 (Nunney & Campbell 1993) up to 10,000 individuals are being voiced as necessary to compensate for genetic, demographic and ecological factors (Mike Lawrence pers. comm.).

When reviewing these concepts for *P. cordata* in Britain it was noted that, between the two clones, there is a maximum of four alleles for any one gene locus and the rarest allele would have a frequency of 0.25. This assumes no novel genetic variation arising through somatic mutation. The minimum viable population figure of 50 individuals is selected to conserve alleles which occur as infrequently in a population as 5% (0.05). The experimental population design, therefore, did not focus on the typical captive breeding numbers but centred on the available allelic diversity especially at the critical *S* locus.

In *P. cordata*, as in its cultivated relatives, self-incompatibility is of the gametophytic type. This is controlled by a single *S* locus at which typically there can be many different *S* alleles within a population (S_1, S_2 etc.). If the single *S* allele carried by a haploid pollen grain matches either of the *S* alleles in the diploid genotype of the style on which it lands, that pollen grain is unable to effect fertilisation (see Richards (1986), chapter 6, for further details). It follows that all individuals must be heterozygous at the *S* locus and that a minimum of three different *S* alleles is required for a population to reproduce sexually.

A study of pollen tube growth within controlled, reciprocal interpopulation cross-pollinations was used in an attempt to assess the allelic diversity at the *S* locus. The results were inconclusive and it is still uncertain whether there are three or four *S* alleles in the two British populations. Given that the loss of one of these alleles could have a significant effect on the future reproductive performance and, therefore, the medium-term conservation of the British representatives of *P. cordata*, the experimental population was designed to accommodate this.

The experimental population

On the assumption that there are four *S* alleles in the two clones, there could be a maximum of six different *S* genotypes in a sexually reproducing population founded from both sources. From a balance of mathematical considerations (to increase the chances of each genotype being equally represented) and practical constraints, a minimum number of ten individuals of each possible *S* genotype was selected, thus indicating a minimum population size of 60 individuals.

To maximise the long-term conservation of the *S* alleles, and any other genetic diversity, it was important to ensure that all individuals would have an equal chance of contributing to the next generation. Where there are three alleles for self-incompatibility, the plants would be semicompatible with three possible offspring genotypes; however, their frequencies would vary and would be dependent on the female (Table 1 (a)). With four alleles the plants would be totally cross-compatible and there would be six possible genotypes including four offspring (Table 1 (b)).

Table 1. Segregations at the S locus in cross-pollinations involving three and four S alleles.

Parents female	male		Offspring	
(a) three S alleles (semicompatible crosses)				
S_1S_2	x	$[S_1]S_3$ [a]	$\frac{1}{2}S_1S_3$	$\frac{1}{2}S_2S_3$
S_1S_3	x	$[S_1]S_2$	$\frac{1}{2}S_1S_2$	$\frac{1}{2}S_2S_3$
(b) four S alleles (fully compatible crosses)				
S_1S_2	x	S_3S_4	$\frac{1}{4}S_1S_3$	$\frac{1}{4}S_1S_4$
S_3S_4	x	S_1S_2	$\frac{1}{4}S_2S_3$	$\frac{1}{4}S_2S_4$

[a] Pollen grains carrying S_1 are unable to effect fertilisation.

In the case of three S alleles, each cross would yield one of the parental genotypes plus the new combination S_2S_3. If this were left unchecked 50% of a seedling-based population would consist of S_2S_3. In order to balance a newly established population, an equal number of seedlings would need to be raised from each female. In addition one-third would need to be parental genotypes propagated vegetatively. Table 2 (a) illustrates the population structure using the minimum number of 60 individuals. For four S alleles the expected frequencies should not vary and would not be dependent on the female. However, the parental genotypes are missing and would need to be added to balance a re-established population as illustrated in Table 2 (b). The elegance of this system is that ten vegetative propagules are required from each parent and 20 seedlings from each reciprocal cross regardless of whether there are three or four S alleles present.

The reintroduction

The choice of the reintroduction site was governed by a number of factors:

- the site must be within the existing range of the species;
- the site must be outside the range of likely cross-pollination with *P. communis*;
- the site must be free from disturbance;
- the climate must be similar to that of the 'native' habitat;
- the soil must be like that of the extant populations (moist and acidic).

Data on climate and soil type (Jackson 1994) were used to evaluate a shortlist of possible reintroduction sites. Planting started in September 1995. To maximise the degree of outcrossing, the planting design is of ten groups of six plants consisting of each parental genotype along with two offspring from each reciprocal cross (Figure 4). A 4 m spacing between the plants was selected to enable the crowns to intermesh to aid cross-pollination by bees. The groups of plants are 20 m apart and cross-pollination should also occur between them.

Table 2. Number of plants required for the experimental population if three or four alleles are controlling self-incompatibility.

(a) three S alleles					
Genotype	Vegetative from parent		Seedlings from cross		Total
	S_1S_2	S_1S_3	S_1S_2 x $[S_1]S_3$	S_1S_3 x $[S_1]S_2$	
S_1S_2	10			10	20
S_1S_3		10	10		20
S_2S_3			10	10	20
Total	10	10	20	20	**60**
(b) four S alleles					
Genotype	Vegetative from parent		Seedlings from cross		Total
	S_1S_2	S_3S_4	S_1S_2 x S_3S_4	S_3S_4 x S_1S_2	
S_1S_2	10				10
S_3S_4		10			10
S_1S_3			5	5	10
S_1S_4			5	5	10
S_2S_3			5	5	10
S_2S_4			5	5	10
Total	10	10	20	20	**60**

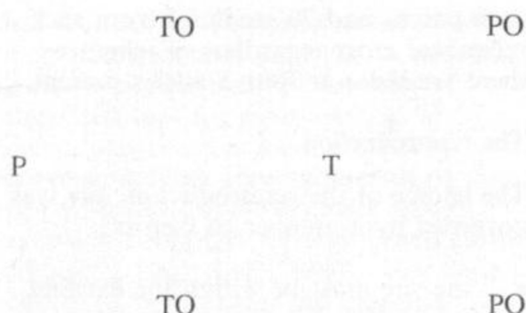

Figure 4. Planting design of a six-plant group in the experimental population (Truro parent (T), Plymouth parent (P), Truro offspring (TO) and Plymouth offspring (PO)).

Each plant will be given a unique number, its parentage recorded and they will be accurately surveyed and mapped. It is hoped that the individual DNA fingerprints of the seedling component of the population can be identified. This would enable future studies on the competitive abilities of each individual as they colonise the site through clonal and sexual reproduction.

Concluding remarks

The combination of a broad RAPD survey of individuals with test hybridisations provided both a biochemical and a biological assessment of genetic variability within *P. cordata*. The biochemically detected genetic differences and those detected on the basis of hybridisation correlate perfectly.

DNA fingerprinting proved to be an important tool for the conservation strategy. The unique information that it provided was used to:

- determine a targeted hybridisation programme which avoided wasting resources on indiscriminate pollinations;
- design the experimental population on sound genetic principles developed for this particular scenario rather than an academic application of wider principles;
- develop sexual and clonal propagation targets, which will avoid over-production and help to balance the contribution of each genotype to the next generation;
- identify the paucity of genetic variation in the British populations and to indicate the need to study diversity within mainland European populations.

Finally, it is not clear whether *P. cordata* came to Britain naturally, or was introduced long enough ago for no records to remain. If a few plants were originally imported and clonal stock planted, there would never have been much genetic variety in the British plants. Alternatively the genetic depauperacy may be a result of long-term population decline.

If the species has been introduced and propagated vegetatively, or has always been present in very low numbers, it is unlikely that any genetic adaptation to local conditions can have happened. Furthermore, with the current two founding clones, the available diversity for long-term adaptation to changes in the environment is small. A full understanding of the genetics of this species can only be gained after a similar study of mainland European populations, data from which should be available in the next five years. At that point a review should be undertaken and must include an assessment of the status of the British populations as well as the possibility of introducing additional variation from the continent.

Genetic considerations will continue to be important to the recovery programme of *P. cordata* for the foreseeable future.

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