GENETIC VARIATION IN *FITZROYA CUPRESSOIDES* CULTIVATED IN THE BRITISH ISLES, ASSESSED USING RAPDS

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Fitzroya cupressoides (Molina) Johnston (*Cupressaceae*), a threatened conifer native to southern South America, has been cultivated in a number of gardens and arboreta in the British Isles since its introduction in 1849. In order to assess the importance of these cultivated trees for *ex situ* conservation, foliar samples were collected from 48 trees from throughout the British Isles, including five of known wild origin (Chile). DNA was extracted from these samples and assessed using the RAPD technique, in order to examine the extent of genetic variation. All samples from the cultivated trees of unknown origin, with one exception, were found to be genetically identical. In contrast, the five samples of known wild origin revealed pronounced polymorphism, varying from 5.3% to 49.1% between individuals. These results suggest that virtually all of the *F. cupressoides* trees currently cultivated in the British Isles have been derived from a single individual by vegetative propagation. Their value for *ex situ* conservation is therefore likely to be extremely limited. The implications of these results for the genetic conservation of other taxa in gardens and arboreta is discussed.

Keywords. Conifer, *ex situ* conservation, genetic variation, RAPDs.

INTRODUCTION

The conifer *Fitzroya cupressoides* (Molina) Johnston (*Cupressaceae*) is a member of a monotypic genus restricted to small areas of the temperate rainforests of southern Chile and adjoining Argentina. This tree species is well known for its size and longevity, with trunk diameters of up to 5m and heights of up to 60m, and maximum age in excess of 3600 years (Lara & Villalba, 1993). As a result of its highly prized and extremely durable timber, populations of *F. cupressoides* (alerce) have been heavily exploited for the last three centuries, and are consequently much reduced in extent. Its slow growth rate and specific regeneration requirements have also restricted its ability to recover after logging. As a result, *F. cupressoides* has been the focus of increasing conservation concern in recent decades. The species is considered as threatened according to the IUCN criteria and is also listed on CITES Appendix I (Golte, 1996) and vulnerable by Farjon *et al.* (1993).

F. cupressoides was introduced into Britain in 1849 through the nursery of Messrs Veitch and Sons, and has been grown in plant collections throughout the British

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Isles ever since. The species is currently represented in at least 60 arboreta and gardens. Such a population of trees could potentially be of value for conservation as an *ex situ* germplasm bank, and as a potential source of material for recovery of degraded populations within the native range of the species. However, in order to assess the value of such a population for conservation, measurements of genetic variation are required (Millar & Libby, 1991).

A number of techniques are now available for assessing genetic variation in trees. The random amplified polymorphic DNA (RAPD) technique (Williams *et al.*, 1990) is a relatively simple method which can generate individual specific 'fingerprints'. These are generated via the polymerase chain reaction using short, random sequence primers. The method has been used successfully in a wide array of genetic studies on plant species including population genetics (Vicario *et al.*, 1995), germplasm identification (Kiel & Griffin, 1994), relationships within and between species (Chalmers *et al.*, 1994) and conservation (Rossetto *et al.*, 1995). It is therefore an ideal technique to reveal variation and genetic relationships within *ex situ* populations with a view to comparison with natural populations. However, RAPDs do have disadvantages in that comparison between laboratories due to poor repeatability has been reported (Penner *et al.*, 1993). We have avoided this problem by performing PCRs in strictly identical conditions in a single laboratory and by only using a subset of repeatable RAPD markers. Also, RAPDs provide dominant marker data and therefore traditional measures of population differentiation and relationships between individuals (such as F statistics, Wright, 1951) cannot be calculated without making assumptions of expected allele frequencies (Lynch & Milligan, 1994). To avoid making possibly incorrect assumptions, simple genetic distance methods have been used (equivalent to simple %polymorphism used here).

The aim of this investigation was to examine the genetic variation within *F. cupressoides* cultivated in the British Isles, to assess their value for *ex situ* conservation of the species. Samples were collected from as many of the cultivated trees within the UK and Ireland as possible, and analysed using RAPDs. This study forms part of a collaborative project involving institutions within both the UK and Chile focusing on the genetic conservation of *F. cupressoides*, including both *in* and *ex situ* approaches, and recovery of degraded populations.

MATERIALS & METHODS

Sample collection

The aim of the sampling programme was to collect from trees of different ages and in different localities to sample the full range of variation present. This initially involved locating and contacting gardens and arboreta with *F. cupressoides* in the their collection, particularly those with older trees. Trees to be sampled were initially identified by obtaining the records of organisations such as the Tree Register of the

British Isles (TROBI), the living collection databases of the National Trusts, botanic gardens and other historic collections of plants. In all, 96 records were received from TROBI, 24 from the National Trust and six from the National Trust for Scotland.

TROBI records provided measurements and other details for 96 trees in 65 different locations. Some of the records were taken from historic records such as the 1931 Conifer Conference. All of the TROBI records represented trees that had been planted before 1960, with the majority dating back to before 1945. Twelve had insufficient information for them to be accurately located (including one tree thought to have been planted in 1859 – Hewel Grange) and another four were already recorded as being dead. Of the remaining records, only five were planted before 1875: Belsay Castle, Northumberland (1852), Pencarrow, Cornwall (1852), Killerton, Devon (1864), Scorrier House, Cornwall (1868), and Powerscourt, Co. Wicklow (1869).

National Trust records provided information on 24 trees, four of which also appeared in the TROBI records. Eighteen of the remainder had been planted since 1980 and most of those since 1988. The original source of these plants was unknown. National Trust for Scotland records included six trees, five planted since 1980 while the sixth (Culzean) was thought to have been planted around 1900 and had been recorded by TROBI. Ten other trees that had not been previously recorded by TROBI were communicated privately from the proprietors of the collections. These were Exeter University, Penjerrick, Headfort, Derreen, Hilliers and Tollyriver Park.

A target list was drawn up after collating these records, eliminating duplicates and trees known to have died. The final list included all living trees thought to have been planted before 1900 and a selection of trees planted after that date (see Table 1). Where planting dates had not been recorded for trees more than 10m high, an estimated planting date was deduced using growth measurements taken from TROBI records for trees with known planting dates. In addition, five accessions of verified known wild origin were selected from trees growing at Younger Botanic Garden, Benmore. These originated from a 1988 collection in Chile at the Alerce Costero National Park (40°10′S, 73°28′W).

A letter explaining the project and requesting samples (20–30g of fresh foliage) was then sent to the owners or head gardeners. Planting dates, measurements and any details about the origin of the plants were also requested. An article outlining the project and requesting samples and information was published in the newsletter published by Plantnet, the umbrella organization for plant collections in the UK and Ireland. A total of 48 trees, including the five accessions of known wild origin, were included in the RAPD analysis (Table 1). Needle tissue was collected and dried in sealed plastic bags containing silica gel (S4883 silica, Sigma Chemical Company Ltd, Fancy Road, Dorset, UK) and stored at 4°C prior to DNA extraction. This method of sample preservation is suitable for maintaining DNA integrity (Chase & Hills, 1991). RAPDs were also performed on DNA isolated from fresh, frozen, and silica dried samples from the same individual tree and identical profiles were obtained (results not shown).

TABLE 1. Details of location and planting date of *Fitzroya cupressoides* trees sampled and included in the RAPD analysis. * denotes samples of known Chilean origin. The planting dates of all but four trees are based on documentary evidence from the gardens involved. For samples 15 and 16, planting date was estimated from their growth (diameter at breast height) and for samples 17 and 18 age was estimated from tree ring counts.

No.	Location	Planting Date	No.	Location	Planting Date
1	Culzean, Ayrshire	c.1900	25	Bicton Park, Devon	c.1911
2	Ardkinglas, Argyll	c.1875	26	Sheffield Park, W. Sussex	1988
3	Tregrehan, Cornwall	c.1875	27	Sheffield Park, W. Sussex	1990
4	Arduaine, Argyll	1985	28	Derreen, Co. Kerry	c.1970
5	Rowallane, Co. Down	c.1950	29	Hilliers, Hampshire	1968
6	Exeter Uni., Devon	1973	30	Hilliers, Hampshire	1965
7	Exeter Uni., Devon	1973	31	Hilliers, Hampshire	1965
8	Exeter Uni., Devon	1973	32	Bodnant, Clwyd	c.1931
9	Cotehele N.T. Devon	1990	33	Fota, Co. Kerry	1988
10	Glenveagh, Donegal, Eire	1985 c.1930	34	Trelissick, Devon	1973
11	High Beeches, W. Sussex		35	Kilmacurragh, Wicklow, Eire.	c.1875
12	Westonbirt, Gloucestershire	1981	36	Tollyriver Park, Co. Dublin, Eire	c.1975
13	Westonbirt, Gloucestershire	c.1970	37	Talbot, Co. Dublin, Eire	1960
14	Penjerrick, Cornwall	c.1985	38	Endsleigh, Devon	c.1910–1920
15	Drymsynie, Argyll	c.1900	39	Bedgebury, Kent	1927
16	Kilmun Arboretum, Argyll	c.1930	40	Wakehurst, Kent	c.1969
17	Younger BG, Argyll, 19588381	c.1900	41	Powis, Powys	c.1930
18	Younger BG, 19588384	c.1910	42	Belsay, Northumberlandd	1856
19	Younger BG, 19658024	c.1965	43	Ardgowan, Renfrewshire	c.1875–1890
20	Younger BG, 19270497	c.1927	44*	Younger BG, 19882735	1988
21	Headfort, Co. Meath	c.1912	45*	Younger BG, 19882735	1988
22	Inverewe, Ross-shire	1963	46*	Younger BG, 19882735	1988
23	Camperdown Park, Fife	c.1900	47*	Younger BG, 19882735	1988
24	Bicton College, Devon	c.1930	48*	Younger BG, 19882735	1988

DNA isolation

The following DNA isolation method was adapted for *F. cupressoides* tissue from that of Doyle & Doyle (1990). 0.5g of dried plant tissue was ground with a mortar and pestle under liquid nitrogen to a fine powder. After allowing liquid nitrogen to evaporate, 5ml of CTAB isolation buffer was added (2% CTAB (cetyltrimethylammonium bromide), 1.4M NaCl, 20mM EDTA, 1% PEG 8000, 100mM Tris-HCl (pH 9.5)) and incubated at 55°C for 1 hour. The mixture was treated twice by shaking with 5ml of chloroform/isoamyl alcohol (24:1) followed by centrifugation to separate phases and removal of the aqueous layer. 5ml of isopropanol was added

to the final aqueous extract and mixed gently to precipitate the DNA. The precipitate was then centrifuged to pellet DNA and washed in 70% ethanol. Finally the DNA was dissolved in 1.5ml TE buffer (10mM tris base, 1mM EDTA, adjusted to pH 8.0 with HCl) and stored at 4°C. DNA concentration was determined by comparison to standards on agarose gels and dilutions made in TE buffer to give 25ng DNA/μl for RAPD reactions.

RAPD reactions

RAPDs were performed using the following conditions per 25ml reaction which were optimised to give repeatable markers: 50ng template DNA, 10 pmol primer, 1 Unit *Taq* polymerase, 200μM each dNTP, 1mM $MgCl_2$, reaction buffer (16M $NH_4)_2$ SO_4, 67mM Tris-HCl (pH 8.8), 0.1% Tween-20. 45 PCR cycles were used consisting of 94°C for 1min, 40°C for 1min, 72°C for 2min and a final extension phase of 72°C for 5min. Forty different random 10-mer primers were screened in the RAPD analysis (Kits OPK and OPAL, Operon Technologies, Alameda, California), nine of which gave the clearest markers for subsequent analysis (Table 2). RAPD products were separated on 8% Acrylamide gels (29:1 acrylamide:bis-acrylamide) and visualized by staining with ethidium bromide and photographed over UV light. RAPD markers were scored in a presence/absence fashion and pairwise percentage polymorphism between samples calculated using:

$$\%\text{Polymorphism} = \frac{S}{N} \times 100$$

where S is the number of shared bands between individuals and N is the total number of marker positions. Only RAPD bands that could unequivocally scored were counted in the analysis. This can be difficult between lanes separated by many others and differing intensities of PCR products. To ensure this, generally only bands in size ranges between clearly visible monomorphic bands were scored. This severely

TABLE 2. Sequence of primers used in RAPD analysis.

Name	Sequence 5′ to 3′
OPAL-09	CAGCGAGTAG
OPAL-12	CCCAGGCTAC
OPAL-17	CCGCAAGTGT
OPAL-18	GGAGTGGACT
OPK-9	CCCTACCGAC
OPK-16	GAGCGTCGAA
OPK-18	CCTAGTCGAG
OPK-19	CACAGGCGGA
OPK-20	GTGTCGCGAG

reduced the potential number of bands that could have been scored but, importantly, avoided mis-scoring.

RESULTS

The nine primers selected differed in the number of markers produced, from five (OPK 19 and 20) to 17 (OPK 16), with an average of 10.3 bands per primer. The 93 RAPD marker positions scored for 42 of the samples of unknown origin were identical. Sample 13 (from Westonbirt arboretum) was exceptional, in that it exhibited 23.7% polymorphic RAPD bands between it and the remaining trees of unknown origin (see Table 3). All primers except OPAL18 (for which all British material bands were monomorphic) revealed polymorphisms in sample 13. The history of this plant is not known and it cannot be ascertained if its genetic difference is due to it originating from a different wild collection. Unfortunately, since this work was carried out the specimen in question has died and therefore no further investigations can be made. 52 of 93 RAPD band positions among known wild origin samples (Nos. 44–48) were polymorphic (56%), nearly twice the variation observed among British samples. Between known wild origin samples percentage polymorphism varied from 5.3% to 49.1% (Table 3). The mean percentage polymorphism between sample 13 and the remaining British trees was similar to that among the accessions of known wild origin (23.7% and 21.2% respectively).

The RAPD patterns typically demonstrated one polymorphism between sample 13 and other British samples (Fig. 1). It was found that when performing PCR on a large number of samples it was difficult to always achieve 100% amplification. Such lanes were therefore treated as 'missing values' which did not affect the analysis of pairwise percentage polymorphism. Initial optimisation of the RAPDs showed patterns to be reproducible between PCRs and because RAPD bands were predominantly monomorphic, also demonstrating reproducibility, replicate RAPD reactions

TABLE 3. Percentage polymorphism between groups of *Fitzroya cupressoides* trees, calculated on the basis of RAPD analysis. (A) refers to the 42 trees which were genetically identical, (B) to Sample 13 (Westonbirt, see Table 1) and accessions of known wild origin (Chile; nos. 44–48, see Table 1).

			Accessions of known wild origin (Chile)				
	A	B	44	45	46	47	48
A	0	23.7	34.4	30.1	33.9	30.1	30.1
B		0	36.6	40.9	49.1	44.1	36.6
44			0	25.8	35.1	28.0	21.5
45				0	5.3	6.5	25.8
46					0	8.8	31.6
47						0	23.7
48							0

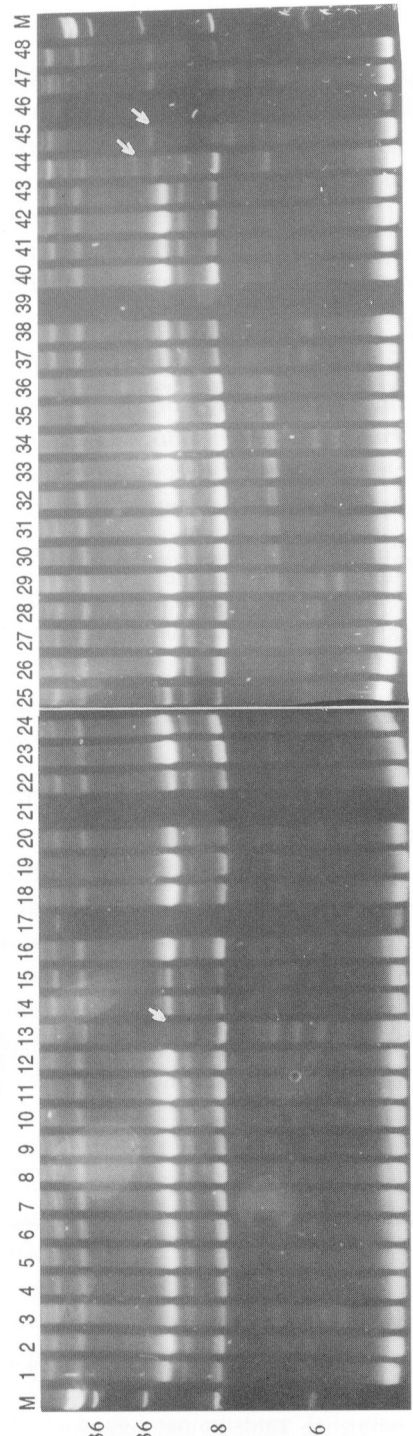

FIG. 1. RAPD products generated by primer OPK-09 for British *F. cupressoides* samples (lanes 1–43) and *ex situ* known Chilean origin samples (lanes 44–48). The arrow in lane 13 indicates one polymorphic band, lacked by that sample. Arrows in lanes 44 and 45 indicate two of the bands polymorphic between known Chilean origin samples which are absent in British samples. Lanes denoted 'M' contain Gibco 1 Kb DNA markers, lengths are shown in base-pairs.

for the entire set of samples were not performed. A UPGMA dendrogram constructed using pairwise percentage polymorphism (Fig. 2), shows that the degree of variation evident between sample 13 and other British trees is of a similar order to that between Chilean trees, indicating that it originated from a different individual from the remaining 42 trees.

DISCUSSION

The history of Fitzroya cupressoides *cultivation in the British Isles*

The first introduction of *Fitzroya cupressoides* to the British Isles was by William Lobb, who collected it during his second expedition to South America in 1847 (Dallimore, 1932). Lobb was also responsible for the introduction of at least three other Chilean conifers (*Podocarpus nubigenus*, *Saxegothaea conspicua* and *Pilgerodendron uviferum*) (Veitch, 1906). The precise origin of his collection of *Fitzroya* has never been fully established: his field notes were either lost, or due to the intense competition between collectors and their sponsors, were not revealed. Correspondence from Lobb to his sponsors (Lindley, 1851) is vague and refers to collections made from a great part of the island of Chiloë, islands of the nearby archipelago and the coast of Patagonia.

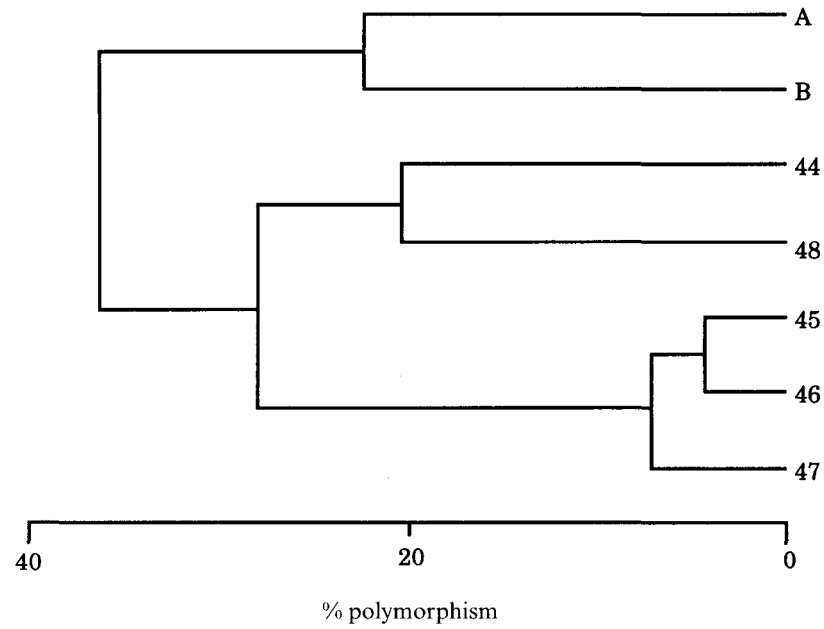

FIG. 2. UPMGA dendrogram showing degree and relationship of RAPD polymorphism between British and Chilean *F. cupressoides*. (A) refers to the 42 trees which were genetically identical, (B) to Sample 13 (Westonbirt, see Table 1) and accessions of known wild origin (Chile; Nos. 44–48, see Table 1).

It is also uncertain whether he collected the species as seed or as cuttings. It is interesting to note that when the species was first offered for sale in 1852, the advertisement placed by Messrs. Veitch and Sons described their stock as plants, not as seedlings which was the usual custom for those new introductions that had been collected as seed (Anon, 1852; Anon, 1851). The following year, two other nurseries offered plants of *Fitzroya cupressoides* for sale (at vastly reduced prices), suggesting that they may also have received material from William Lobb. There is no evidence that other collections were made at that time and dispersed throughout the British Isles and it is likely, given their identical genotypes revealed in this study, that all British trees except recent Conifer Conservation Programme (CCP) collections originated from material gathered by William Lobb. Evidence of the dispersal of this material around the British Isles is not available except by inference from planting dates.

Implications of molecular analyses

The results of the RAPD analysis indicated that all except one of the cultivated trees of unknown origin were genetically identical, and that the 43 trees represent only two different genotypes. This suggests that the trees were vegetatively propagated from the original introductions. This result was entirely unexpected, as it was assumed at the outset that the species was originally propagated by seed when it was introduced. However, observations made during collection of the samples used in this study indicated that the trees had an almost identical growth habit and markedly similar foliage. This is despite pronounced variation in growth conditions, with samples collected from trees growing in Cornwall, England, Ireland, Wales and Scotland. All trees had a pendulous habit without a dominant leader, often a sign of vegetative propagation in conifers (Hartmann & Kester, 1975). *F. cupressoides* trees in the wild usually have an upright, conical habit throughout their life (J. Armesto, Universidad de Chile, pers. comm.). Collections taken from across the natural range of the species show large variation in morphological characters (P. Thomas & M. Gardner, personal observation), and molecular analysis of material collected from a wild population (samples 44–48) indicates a high degree of genetic variation within populations. A clonal origin of cultivated trees of *F. cupressoides* is also supported by the fact that many produce large numbers of female cones but none have ever been known to produce viable seed; the absence of males has been a source of comment almost since the time of its introduction (Lindley, 1851; Elwes & Henry, 1910; Doyle & O'Leary, 1934). Vegetative multiplication may have therefore been the only option, once the original introductions had been received.

It is unknown how many genotypes were introduced by the original introduction. *F. cupressoides* seed production can be unpredictable, varying in number and viability from year to year. The original collection of *F. cupressoides* by William Lobb was therefore most likely to have been cuttings. It is also possible that collection of seed was made but germination and survival rates were low, and subsequent propagation

and horticultural selection reduced the variation to two genotypes. Recent observations that *F. cupressoides* can grow naturally in clonal groups as a result of suckering (S. Fraver, personal observations) could also have resulted in a lack of genetic diversity in the material originally introduced.

Implications of results for ex situ *conservation*

The surviving trees of William Lobb's collection are in effect a single clone, the tree from which sample 13 was taken having died since the sample was collected. The value of these trees to *ex situ* conservation of the species is consequently very limited. The paucity of variation in this material is emphasized by the high degree of polymorphism recorded in the relatively recent accessions of Chilean origin included in this study. Clearly, additional material needs to be introduced into the population of *F. cupressoides* within Britain, if it is to be of value to conservation efforts. A priority would be the establishment of male trees, which would enable the development of a breeding population, as has occurred with some other Chilean conifers introduced to Britain, such as *Podocarpus salignus* and *Araucaria araucana* as a part of the RBGE CCP. As a result of recent collecting, the CCP currently holds 79 *F. cupressoides* genotypes which are growing well in nursery conditions with plans for their eventual planting as an *ex situ* resource in the UK. The lack of genetic variation in the British Isles' *F. cupressoides* prior to the CCP's efforts emphasizes the importance of the programme in increasing the genetic base of the species in cultivation and the value of the wild stocks as the primary conservation objective. This study has shown that the five 1988 collections of *F. cupressoides* exhibit genetic variation, comparable to that observed in recent studies of natural *F. cupressoides* (unpublished data). These 79 genotypes together with future collections will therefore form an important resource for *F. cupressoides* conservation.

The implications of these results with respect to the value of *F. cupressoides* cultivated in the British Isles are not entirely negative. A single genotype of this threatened species has survived in cultivation for over 150 years and there are sufficient young plants to ensure its survival for another 150 years. This demonstrates that it is possible for woody plants to be maintained in cultivation over a period of centuries. However, the role of private landowners and gardeners in the past with respect to conservation of genetic diversity has been positive. The dispersal of material over many sites offering a form of insurance against the loss of individual genotypes may have been successful but lack of attention to the genetic diversity of the material originally introduced and the common use of clonal propagation in horticultural methods has reduced genetic diversity.

The results of this study have wider implications for the *ex situ* conservation of plants. *Ex situ* genetic conservation of threatened species is increasingly being used as a justification for botanic gardens and arboreta (BGCI, 1996) The importance of *ex situ* conservation was explicitly recognized by the Biodiversity Convention ratified at UNCED in Rio de Janeiro, 1992 (UNEP 1995). Article 9 of the Convention

referred to the need for establishing and maintaining facilities for the *ex situ* conservation of plant species. Yet in many cases (such as with *F. cupressoides*) detailed information on the origin, and the subsequent history of the introduction, are almost completely lacking in public and private botanic gardens. It is conceivable that many other introductions have a very narrow genetic base, and may therefore have very restricted value for conservation. The results of the present study suggests that claims for the conservation value of *ex situ* collections should therefore be viewed with caution, unless supported by the kind of genetic analysis described here.

Remarkably few studies of genetic variation in *ex situ* collections from a conservation standpoint are available for comparison. However, the current results concur with those of other studies that RAPDs can provide valuable genetic information on germplasm collections. Other studies have examined commercial crop species germplasm, such as rice (Virk *et al.*, 1995), *Eucalyptus* (Kiel & Griffin, 1994) and *Brassica* (Kresovich *et al.*, 1992). In particular, this work has highlighted the usefulness of RAPDs for detection of clones in collections. RAPDs have also be used for evaluation of diversity in natural populations e.g. *Theobroma* (Russel *et al.*, 1993), where the information provided allows the development of collection strategies that preserve genetic diversity and structure observed in populations (Schaal *et al.*, 1991). In *F. cupressoides* RAPDs will therefore be very useful in the future to assess diversity of material already collected by the CCP and to formulate reintroduction plans for the species from native and *ex situ* germplasm.

ACKNOWLEDGEMENTS

This study forms part of a collaborative project between the Conifer Conservation Programme (CCP), based at the Royal Botanic Garden Edinburgh (RBGE), and the Universities of Edinburgh, Austral de Chile and Chile. The study was partly funded by a grant from the Darwin Initiative for the Survival of the Species, and a Royal Society grant to ACN. We are extremely grateful to all the people who so generously provided samples for analysis. Thanks also to Dr Juan Armesto (Universidad de Chile), Dr Antonio Lara (Universidad Austral de Chile) and Shawn Fraver for assistance with fieldwork and for valuable discussions about alerce, the Tree Register of the British Isles (TROBI), The National Trust and The National Trust for Scotland.

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Received 4 September 1997; accepted in revised form 14 April 1998