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Genetic variation in *Fitzroya cupressoides* (alerce), a threatened South American conifer

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Abstract

***Fitzroya cupressoides* (alerce, Cupressaceae) is a large and exceptionally long-lived conifer, endemic to a restricted area of southern Chile and neighbouring areas of Argentina. As a result of its high economic value, the species has been severely exploited for timber, and remnant populations are fragmented and often highly disturbed. The species is thought to have undergone a major range contraction during the last glaciation. In order to assess the extent of genetic variation using DNA markers within and between populations of this species, samples were obtained from throughout the natural range and analysed for random amplified polymorphic DNA (RAPD) variation. Eight 10-mer and three 15-mer primers were used to produce a total of 54 polymorphic bands. Shannon's diversity estimates were calculated to provide an estimate of the degree of variation within each population. Values varied from 0.343 to 0.636 with only the lowest value differing significantly from the others (S_{pop} = 0.547). This indicated that there is a significant degree of variation within each population, and did not provide evidence for genetic 'bottleneck' effects within the species. A pairwise distance measure calculated from the RAPD data was used as an input for principal coordinate (PCO) and AMOVA analyses. The first three principal coordinates of RAPD distances described 8.3, 5.9 and 5.4% of the total variance, respectively, and a degree of clustering of samples according to their geographical origin was detectable. AMOVA analysis indicated that although most of the variation (85.6%) was found within populations, a significant proportion ($P < 0.002$) was attributable to differences between populations. An UPGMA dendrogram constructed using Φ_{ST} values derived from AMOVA produced a pattern broadly similar to that produced by the PCO, highlighting differences between three main groups of populations within Chile: those from the northern Coastal Range, the southern Coastal Range and Central Depression, and the Andes. Populations from Argentina also emerged as significantly different from those in Chile. These results are interpreted in the context of the postglacial history of the species, and their implications for the development of conservation strategies for *Fitzroya* are discussed.**

Keywords: alerce, conservation biology, *Fitzroya cupressoides*, genetic variation, RAPDs

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Introduction

A large number of studies have now been completed assessing the pattern and distribution of genetic variation within tree species. Results indicate that trees tend to display a relatively high degree of genetic variation,

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both within species and within populations, compared with other plant taxa (Hamrick *et al*. 1992). In addition, species with widespread distributions, outcrossing breeding systems, and the potential for long-distance gene flow tend to display a relatively high degree of intraspecific variation, compared to species with other combinations of traits (Hamrick *et al*. 1992). As conifers generally display some if not all of these traits, it is understandable that most studies of coniferous trees have identified high genetic variability, and a relatively low degree of population differentiation (Hamrick *et al*. 1979, 1992; Loveless & Hamrick 1984; Ledig 1986). In contrast to this general pattern, relatively low genetic diversity has been recorded within a number of coniferous taxa, such as *Pinus resinosa* (Fowler & Morris 1977), *P. torreyana* (Ledig & Conkle 1983) and *Abies bracteata* (Ledig 1987). As the latter two species both have restricted ranges, their low genetic diversity supports the suggestion that the genetic variation of endemic species may be reduced by the effects of genetic drift in small populations (Hamrick *et al*. 1992).

Virtually all of the studies of genetic diversity in conifers have been undertaken with north-temperate species (NRC 1991); tropical and south-temperate taxa have received limited attention to date. A study of *Fitzroya cupressoides* using isozymes indicated that its life-history characteristics such as its longevity, polyploidy, and wind dispersal of both pollen and seed contribute to the maintenance of polymorphisms comparable to other long-lived woody endemics (Premoli 1998). In southern Australia, an investigation of the podocarp *Lagarostrobos franklinii* indicated relatively low genetic diversity, which was attributed to a high degree of inbreeding as a result of vegetative reproduction and limited dispersal, and the possible occurrence of range contractions in the past (Shapcott 1991; Gibson *et al*. 1995). Similarly, low genetic variability has been recorded in the New Zealand podocarp *Halocarpus bidwillii*, which was again attributed to past reductions of population sizes leading to evolutionary 'bottleneck' effects (Billington 1991). In the case of New Zealand conifers, it has been suggested that they may generally display lower genetic variability than those from the northern hemisphere, partly because of such historical reductions in population size, and their generally more-restricted distributions (Hawkins & Sweet 1989).

Most previous investigations of genetic variation within tree species, such as those reviewed by Hamrick *et al*. (1992), have been based on the use of isozymes. Although isozyme loci are relatively easy to screen, and have particular value for estimating gene frequencies and heterozygosity, they represent a limited number of coding regions and may therefore not be indicative of the genome as a whole (Schaal *et al*. 1991). In recent years, a wide variety of techniques have become available which can be used to assess variation of DNA directly, such as restriction fragment length polymorphisms (RFLP) (Miller & Tanksley 1990) and methods based on the polymerase chain reaction (PCR), such as microsatellites (Weber & May 1989), PCR–RFLP (Jarvis *et al*. 1994) and randomly amplified polymorphic DNA (RAPD) (Williams *et al*. 1990). The application of such techniques to tree species has highlighted a higher degree of intraspecific variation than was expected on the basis of previous isozyme analyses, and has emphasized the importance of recent evolutionary history in shaping current patterns of intraspecific variation (Newton *et al*. 1998). RAPD has proved to be a particularly valuable technique in this context, reflecting the wide availability of commercial primers and the lack of any need for DNA sequencing information prior to analysis (Gillies *et al*. 1997).

In this investigation, we describe the application of RAPDs to the assessment of genetic variation in *Fitzroya cupressoides* (Mol.) Johnst. (alerce, Cupressaceae), a threatened South American conifer. The species is endemic to southern Chile and neighbouring parts of Argentina, ranging from 39°50′S to 42°30′S in the Coastal Range of Chile, and reaching slightly further south in the Andes, both on the Chilean and Argentinian sides (Veblen *et al*. 1995). The entire range of the species therefore occurs within an area of $\approx 360 \times 200$ km. Throughout this area, populations of alerce are highly fragmented and discontinuous (Veblen *et al*. 1995), the most extensive being located in the Chilean Andes towards the southern end of its range. Extensive populations of *Fitzroya* previously existed in the Central Depression of Chile, situated between the Coastal and Andean mountain ranges, in the area between where the towns of Osorno and Puerto Montt are now located (Donoso 1983; Golte 1996). These populations were extensively logged and cleared by fire for agriculture by European colonists, most notably during the mid-19th century, in what has been described as one of the most rapid and massive deforestations ever recorded in Latin America (Veblen *et al*. 1976). Large numbers of cut and burnt stumps sill remain in this area, giving an indication of the previous extent of these forests (Armesto *et al*. 1995).

Alerce is an impressively large tree, reaching up to 5 m in diameter and over 50 m in height (Veblen *et al*. 1976; Lara 1991). Individuals are generally very slow growing (< 1 mm/year radial increment; Veblen *et al*. 1995) and may also be extremely long lived. On the basis of ring counts, an age of over 3600-years has been ascertained for one individual (Lara & Villalba 1993). Similar to most other conifers, alerce is wind-pollinated and the small (2–3 mm) seeds are wind-dispersed; although the species is generally dioecious it can occasionally be monoecious (Rodríguez *et al*. 1983). *Fitzroya* generally occurs in a variety of vegetation types at altitudes between 100 and 1200 m, usually on acidic, nutrient-poor or somewhat waterlogged

soils, on sites receiving an annual mean precipitation of between 2000 and 4000 mm (Veblen *et al*. 1995).

As a result of its restricted range and a perceived lack of regeneration, the species was considered to be relictual and undergoing a natural decline (Kozdon 1958; Schmithüsen 1960). However, recent research on the population ecology of the species has indicated that the seedings are shade intolerant, but are able to regenerate successfully on sites subjected to episodic disturbance, such as volcanism and landslides (Lara 1991; Donoso *et al*. 1993), low-intensity fires (Armesto *et al*. 1995; Veblen & Ashton 1982; Veblen *et al*. 1995) and treefall gaps (Donoso *et al*. 1993). The great longevity of alerce enables it to persist in areas where such disturbance is very infrequent (Veblen *et al*. 1995). However, the species apparently does not regenerate well after logging, for reasons that are not well understood (Veblen *et al*. 1995).

F. cupressoides is considered to be an evolutionarily ancient taxon, and is was probably much more widely distributed in the past; fossilized fragments of *Fitzroya* wood have recently been isolated from Oligocene sediments in northwestern Tasmania (Hill & Whang 1996). The recent evolutionary history of *Fitzroya* appears to have been very profoundly affected by Pleistocene glaciations. The distribution of genetic variability by means of isozymes has been used to elucidate recent evolutionary history of South American species of *Nothofagus* (southern beech) and *F. cupressoides* (Premoli 1994a; 1997; 1999). Those studies indicated that, for taxa biogeographically restricted to temperate areas, history has played an important factor shaping their gene pools (Premoli 1994b). Evidence from pollen analyses suggests that during the last ice age, South American temperate forests were reduced to very small refugia at various coastal locations in south-central Chile (Markgraf *et al*. 1995); most of the Chilean Central Depression and the Andes south of 38° were covered by an ice sheet at this time (Villagrán 1991). Following the retreat of the glaciers from their maximum around 18 000 years before present (BP), populations of tree species expanded from their glacial refugia (Villagrán 1991). The precise pattern of postglacial migration of *Fitzroya* is difficult to elucidate, as pollen types cannot readily be differentiated from other members of the Cupressaceae, such as *Pilgerodendron uviferum*. However, between 12 500 and 9500 BP these conifers appear to have been concentrated within Chile in the Coastal Range, subsequently migrating to the north and east. These pollen data suggest that *Fitzroya* has only been present at moderate to high elevations in the Andes during the last few years (Villagrán 1991). The recent history of the species in Argentina is less clear; in particular it is unknown whether glacial forest refugia persisted on the eastern side of the Andes. However, a recent study using isozyme markers has hypothesized a multiple refugia scenario during Pleistocene events for *Fitzroya* located on both western and eastern slopes of the Andes (Premoli 1998).

As a consequence of its recent decline and the impact of exploitation, *F. cupressoides* is now considered to be endangered according to the IUCN Red List criteria (Oldfield *et al*. 1998); it is also considered nationally threatened within Chile (CONAF 1989). Logging prohibitions were introduced in Argentina in 1973 and in Chile in 1976, and in 1973 *Fitzroya* was listed on Appendix I of the Convention on International Trade in Endangered Species (CITES), under which international trade is prohibited except for noncommercial purposes. Although the importance of information on genetic variation for developing conservation strategies for threatened species is widely appreciated (Holsinger & Gottlieb 1991), very few such data are available for *Fitzroya*. A survey of foliage terpenes indicated no geographical variability in monoterpene composition, but slight variation in distribution of sesquiterpenes (Cool *et al*. 1991).

The aim of the present investigation was to assess the extent of genetic variation within and between populations of *F. cupressoides* sampled from throughout its natural range. Given the extreme reductions in range which the species appears to have experienced during the Pleistocene glaciations, we tested the following hypotheses: (i) that the extent of genetic variation within the species as a whole would be relatively low, as a result of bottleneck effects; and (ii) that the extent of population differentiation would be higher than expected for a wind-dispersed, wind-pollinated tree species, as a result of genetic divergence between refugial populations. In addition, we assessed the implications of these analyses for the development of conservation strategies for the species.

Materials and methods

Sample collection

The precise status and distribution of *Fitzroya cupressoides* is somewhat uncertain, as no detailed survey has recently been undertaken. Although a variety of different distribution maps have been presented (Lara 1991; Donoso 1993; Donoso *et al*. 1993; Parker & Donoso 1993; Armesto *et al*. 1995; Veblen *et al*. 1995; Golte 1996), the information they provide on the number and location of remaining populations is somewhat contradictory. For example, although extensive populations are known to have existed formerly in the Central Depression of Chile (Golte 1996), there is general consensus among current distribution maps indicating that these populations are now entirely extinct. However, as a result of survey work undertaken as part of this project, a number of small, relict stands were located in this area, and included in the sampling programme. In addition, although the distribution of *Fitzroya*

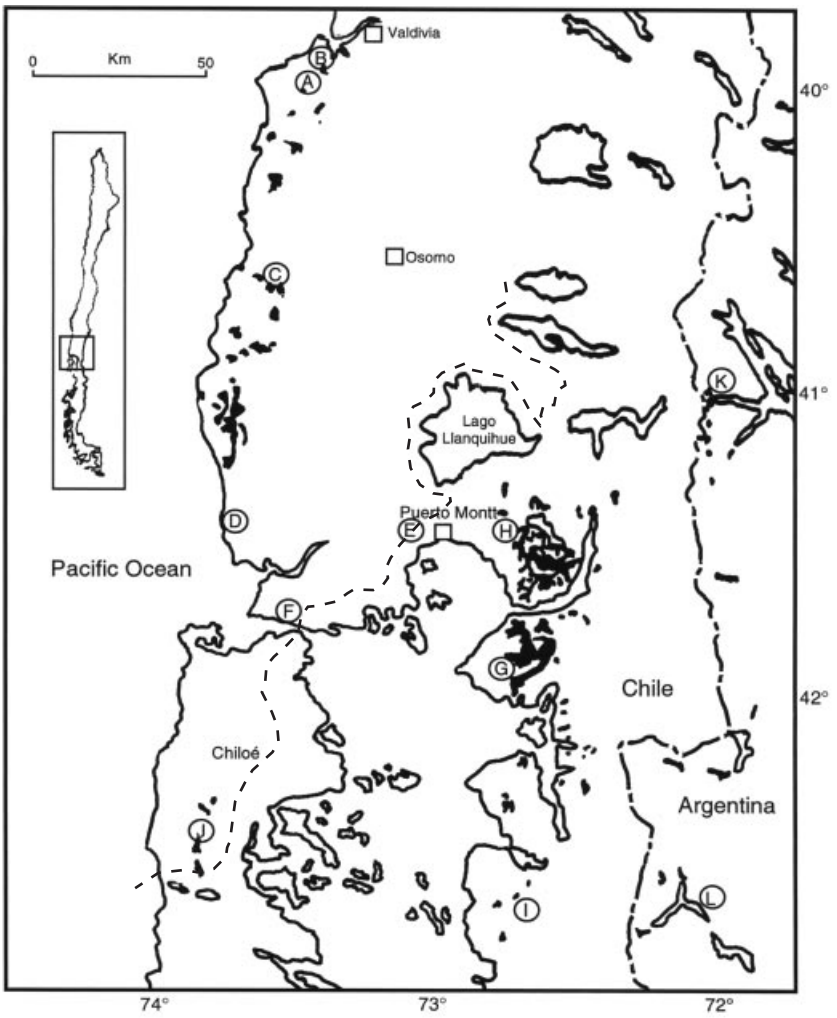

Fig. 1 Sketch map showing the current distribution of *Fitzroya cupressoides* (black, filled areas) and the position of populations sampled during this investigation (A–L; see Table 1 for details). The distribution map is based on that provided by Donoso (1993), on which some of the populations sampled are not marked (see text). The dashed line marks the supposed limit of the ice sheet during the glacial maximum (after Villagrán 1991).

in Argentina has been considered marginal in comparison to that in Chile, recent mapping is indicating that its range on eastern slopes of the Andes may be more extensive than previously beleived (T. Kitzberger *et al*. unpublished).

The aim during the sampling was to include material from throughout the entire distributional range of the species (Fig. 1). Leaf samples were obtained from a total of 89 trees from 12 populations (Table 1). Trees for sampling were selected randomly within each population, but were separated by a minimum distance of 100 m to minimize the chances of sampling closely related or genetically identical individuals (vegetative propagation of *Fitzroya* has been reported in a number of natural stands; Veblen *et al*. 1995). For most populations, eight individuals were sampled (Table 1), but in Cerro Mirador and East Chincay only five and four samples were obtained, respectively, owing to the low number of trees and very difficult access. Limiting the total number of samples to 89 enabled RAPD analysis to be undertaken on a minimized number of gels (four per primer) and PCR runs (one), which could be run in a single tank and a single PCR thermocycler, hence reducing error in comparison of RAPD profiles across nonuniform gels or PCR temperature profile fluctuations. Needle tissue was collected and dried in sealed plastic bags containing silica gel (S4883 silica, Sigma Chemical Co. Ltd, UK) and stored at 4 °C prior to DNA extraction. This method of sample preservation is suitable for maintaining DNA integrity (Chase & Hills 1991). We performed RAPD analysis on DNA isolated from fresh, frozen, and silica-dried samples from individual trees of this species and identical profiles were obtained (results not shown).

DNA isolation

The following DNA isolation method was adapted for *F. cupressoides* tissue from that of Doyle & Doyle (1990). A total of 0.5 g of dried plant tissue was ground to a fine powder with a mortar and pestle, under liquid nitrogen. After allowing liquid nitrogen to evaporate, 5 mL of CTAB isolation buffer was added (2% CTAB

Table 1 Names, locations and altitudes of populations of *Fitzroya cupressoides* sampled for RAPD analysis

Population name	Country	Region	Sample no.	Latitude	Longitude	Altitude (m)
A. Cerro Mirador	Chile	Northern Coastal Range	1–5	40°11′00	73°25′00	450
B. Corral	Chile	Northern Coastal Range	6–13	40°00′00	73°30′00	600
C. East Chincay	Chile	Northern Coastal Range	14–17	40°36′60	73°04′04	448
D. Estaquillas	Chile	Southern Coastal Range	18–21	41°25′06	73°46′00	50
			22–25	41°25′26	73°47′00	50
E. Near to	Chile	Central Depression	26–29	41°26′70	73°07′41	50
Puerto Montt			30–33	41°22′00	72°48′30	50
F. Astillero Central Depression	Chile	South Coastal/	34–41	41°45′	73°34′	35
G. Volcan Hornopiren	Chile	Andean	42–45	41°52′02	72°24′53	1050
			46–49	41°53′31	72°23′38	70
H. Correntoso, Alerce Andino	Chile	Andean	50–53	41°25′	72°36′	450
			54–57	41°30′56	72°37′00	450
I. Chaiten	Chile	Andean	58–59	42°42′22	72°34′35	600
			60–65	42°40′16	73°34′30	600
J. Cordillera de Piuché	Chile	Chiloé Island	66–68	42°22′49	74°02′50	600
			69–73	42°22′	74°02′	570
K. Puerto Blest, Nahuel Huapi	Argentina	Andean	74–81	41°01′76	71°48′97	750
L. Lago Menendez	Argentina	Andean	82–89	42°43′59	71°45′28	500

(cetyltrimethylammonium bromide), 1.4 M NaCl, 20 mM EDTA, 1% PEG 8000, 100 mM Tris-HCl (pH 9.5)) and incubated at 55 °C for 1 h. The mixture was treated twice by shaking with 5 mL of chloroform/isoamyl alcohol (24:1) followed by centrifugation to separate phases and removal of the aqueous layer. A volume of 5 mL 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 1.5 mL of TE buffer (10 mM Tris base, 1 mM 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 25 ng/μL DNA for RAPD reactions.

Table 2 RAPD primer sequences

Name	Sequence 5′ to 3′
OPK16	GAGCGTCGAA
OPK19	CACAGGCGGA
OPK20	GTGTCGCGAG
OPAL06	AAGCGTCCTC
OPAL08	GTCGCCCTCA
OPAL09	CAGCGAGTAG
OPAL12	CCCAGGCTAC
OPAL14	TCGCTCCGTT
TAK03	CCAGCTTAGGGCAAA
TAK18	CCTAGTCGAGCCTAA
TAL17	CCGCAAGTGTAGCTA

RAPD reactions

RAPD reactions were performed using the following conditions per 10 μL reaction which were optimized to give repeatable markers. Reactions were performed in a Perkin-Elmer GeneAmp 9700 thermal cycler: 50 ng of template DNA, 10 pmol primer, 1 Unit *Taq* polymerase, 200 μM each dNTP, 1.5 mM $MgCl_2$, reaction buffer (16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8), 0.1% Tween-20). Forty-five PCR cycles were used consisting of 94 °C for 1 min, 42 °C (10-mer primers) or 56 °C (15-mer primers) for 1 min, 72 °C for 2 min and a final extension phase of 72 °C for 5 min. During preliminary RAPD studies of *F. cupressoides* 10-mer primers often produced very high numbers of bands when visualized on acrylamide gels, which increased the difficulty of scoring bands. In this study random 15-mer primers were also tested in an attempt to produce simpler band patterns. Two 10-mer primer kits, OPK and OPAL (Operon Technologies, Alameda, CA, USA), consisting of 20 primers each, and six 15-mer primers were screened for their ability to produce scorable RAPD markers. From these, a subset of five 10-mers (OPK19, OPK20, OPAL06, OPAL09 and OPAL12) and three 15-mers (TAK03, TAK18 and TAL17) were used in this study (see Table 2 for sequences). The entire sample set was run in duplicate for primers OPK16, OPAL8 and OPAL14, and in each case the scored marker positions were identical. RAPD products were separated on 6% acrylamide gels (29:1 acrylamide:bis-acrylamide) and visualized by staining with ethidium bromide and photographed over UV light.

Only RAPD bands that could be unequivocally scored were counted in the analysis. This can be difficult between lanes separated by many others with differing intensities of PCR products. Generally only bands in size ranges between or close to clearly visible monomorphic

bands were scored. This greatly reduced the potential number of bands that could have been scored but, importantly, avoided mis-scoring of bands for which comigration could not be certain.

Data analysis

RAPD markers were treated throughout as 'genetic' phenotypes. The mating system of *F. cupressoides* in the populations studied is not known and therefore calculation of allele frequencies by compensating for deviation from Hardy–Weinberg equilibrium was not attempted as has been described elsewhere (Lynch & Milligan 1994). In the absence of more-detailed characterization of RAPD bands, their use as genetic phenotypes would appear prudent. Genetic distance methods were therefore used.

A simple pairwise distance measure was calculated from the binary RAPD data using the formula (Sneath & Sokal 1973):

$$D = 1 - (S_{ij}/T_{ij})$$

where S is the total number of shared present band positions and T is the total number of band positions between the ith and jth individuals. These distances were used to perform principal coordinate analysis (PCO) (Gower 1966) which provides a graphical representation of the RAPD relationships between individuals. Population structure within and among populations was analysed using the above distances as input for AMOVA (analysis of molecular variance, Excoffier *et al*. 1992). The AMOVA programme generates 'Φ' statistics analogous to Wright's F_{ST} (Wright 1951) and therefore allows comparison of results to other studies. Shannon's diversity estimates (Lewontin 1972) were calculated to provide a relative estimate of the degree of variation within each population by using the formula:

$$S = -\Sigma p_i \log_2 p_i$$

where p_i is the frequency of presence or absence of each RAPD band (treating each RAPD band as a single locus according to Lewontin 1972). Note that we here denote Shannon's measure as '*S*', not '*H*' so as to avoid confusion with other measures of diversity such as heterozygosity, to which Shannon's is not comparable. S_{pop} was calculated as the mean value of S over all the populations sampled.

Results

A total of 54 polymorphic bands was scored. Each individual sampled gave a unique RAPD band profile, indicating that none of the samples represented repeated samples from the same clone. Calculated pairwise distances were used as input for PCO and AMOVA analysis. No population-specific bands were observed in the data set.

The first three principal coordinates of RAPD distances described 9.3, 7.6 and 6.4% of the total variance, respectively. When the two principal coordinates explaining the most variation were considered, individuals plotted as a broad scatter, with no population clustering independently from all of the others (Fig. 2). However, a degree of clustering of samples according to their geographical origin was detectable. For example, those samples from the northern Coastal Range in Chile (populations A and B)

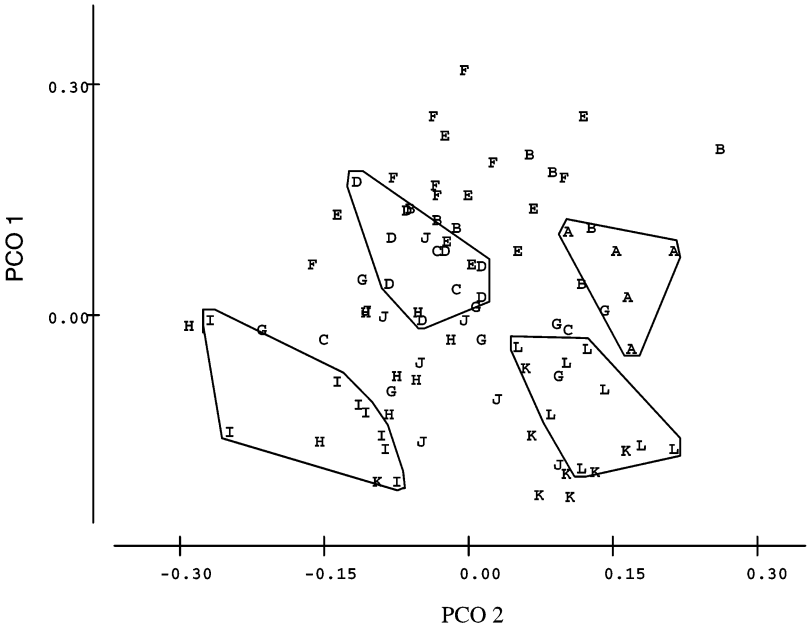

Fig. 2 Plot of the first two principal coordinates for each *Fitzroya cupressoides* individual (describing 14.21% of total variation). Populations are denoted A to L according to Table 1. Four populations, A, D, I, and L, which do not overlap in the graph-space and belong to significantly different groups in the cluster analysis of AMOVA Φ_{ST} values (Fig. 2) are outlined.

Table 3 AMOVA analysis of RAPD variation for 12 *Fitzroya cupressoides* populations. The *P*-value was calculated by 500 replication bootstrap between populations

	d.f.	Sum of squares	Mean squares	Variance component	% of total variance	*P*
Among populations	11	5.833	0.530	0.040	14.38	< 0.002
Within populations	77	18.211	0.237	0.237	85.62	

did not overlap at all with the Andean populations (G, H, I, K, L) or Chiloé (J). In addition, although populations from the Argentine Andes (K, L) shared some overlap with the Andean populations from Chile (G, I, J), there was no overlap between the former and any of the populations from the Chilean Coastal Range or Central Depression (A–F).

The partitioning of genetic variation was further examined by AMOVA (Table 3). Although most of the variation (85.6%) was found within populations, a significant proportion ($P < 0.002$, tested using a 500 replication bootstrap) was attributable to differences between populations. All except three pairwise Φ_{ST} values (J to K, L to K and C to D) derived from AMOVA were significant ($P < 0.05$) when individual pairs of populations were compared (Table 4). When Φ_{ST} values were used to construct an UPGMA dendrogram in order to examine relationships between populations (Fig. 3), a pattern was obtained which was broadly similar to that produced by the PCO. The most striking result was the pronounced difference between the northern coastal populations (A and B) and the others; the Argentinian populations also emerged as relatively distinct (Fig. 3). An unusual and unexpected feature was the clear isolation of the southern coastal population, Astillero. This population is not isolated by any geographical barrier from other coastal or central depression populations.

The relative degree of diversity in each population as measured by Shannon's index varied from 0.349 (Cerro Mirador) to 0.648 (Volcan Hornopiren) (Table 5). The only value significantly different ($P < 0.02$) from the others was that of Cerro Mirador. However, it is possible that this difference is due to the smaller sample size of this population which can bias Shannon's index (Lewontin 1972). The mean diversity for all populations (S_{pop}) was 0.547 (± 0.045, 95% confidence limits).

Discussion

At the outset of this investigation, we hypothesized that *Fitzroya cupressoides* may demonstrate very little intraspecific variation, as a result of the severe range contractions which the species appears to have experienced during the Pleistocene glaciations and consequent 'bottleneck' effects (cf. Barrett & Kohn 1991). The losses of genetic variation which are thought to occur in small, isolated populations have long been understood in theory, and are largely attributable to the effects of genetic drift (Barrett & Kohn 1991). Evidence for low genetic diversity resulting from a historical constriction in population size is available for a number of tree species. For example, analysis of *Pinus torreyana*, which currently numbers fewer than 10 000 individuals distributed between two populations, indicated that each population is composed of identical homozygous genotypes at 59 isozyme loci (Ledig & Conkle 1983). Studies of *Pinus resinosa* have detected almost no intraspecific variability, despite the species being widespread throughout northern USA and southern Canada.

Table 4 Pairwise Φ_{ST} values calculated by AMOVA illustrating differences between populations of *Fitzroya cupressoides*. Φ_{ST} values are given below the diagonal, and the associated *P*-values are given above the diagonal. A–L refer to the populations sampled (see Table 1)

	A	B	C	D	E	F	G	H	I	J	K	L
A	—	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
B	0.0958	—	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
C	0.1594	0.1160	—	0.1916	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
D	0.2064	0.1259	0.0374	—	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
E	0.1829	0.1187	0.0861	0.1159	—	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
F	0.2397	0.1383	0.1770	0.1070	0.1591	—	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
G	0.1334	0.1251	0.0512	0.0829	0.0930	0.1464	—	0.0000	0.0000	0.1537	0.0000	0.0000
H	0.2117	0.1540	0.0986	0.1284	0.1478	0.1749	0.0627	—	0.0000	0.1557	0.0000	0.0000
I	0.2530	0.2233	0.1146	0.1631	0.2296	0.2779	0.1094	0.0879	—	0.0000	0.0000	0.0000
J	0.1392	0.1204	0.1081	0.1214	0.1613	0.1584	0.0434	0.0187	0.0857	—	0.0000	0.0000
K	0.2402	0.2192	0.1522	0.1767	0.2184	0.2724	0.0878	0.1004	0.1644	0.0903	—	0.0000
L	0.1273	0.1698	0.1195	0.1406	0.1880	0.2449	0.0853	0.1386	0.1671	0.1196	0.0614	—

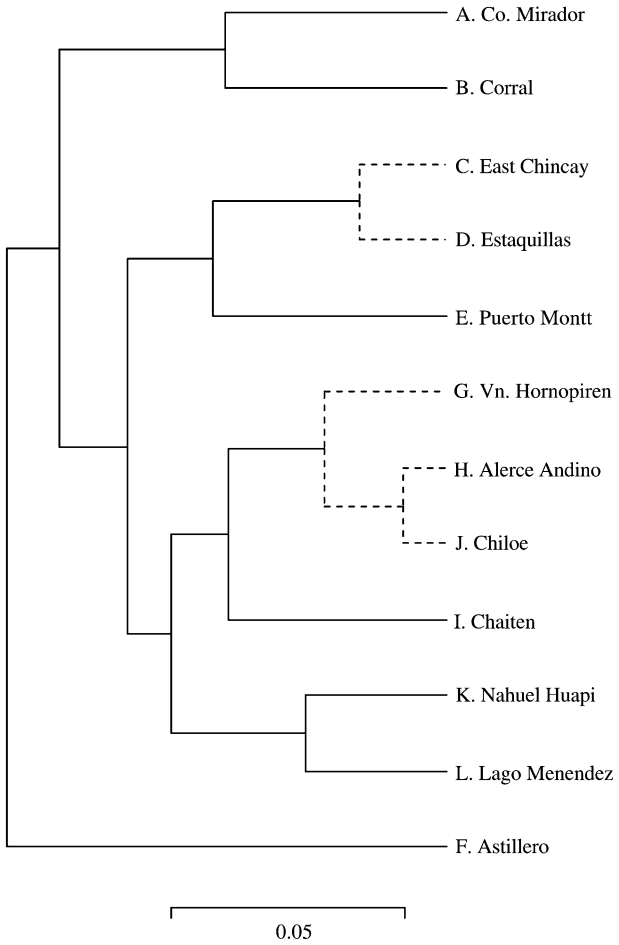

Fig. 3 UPGMA dendrogram constructed using pairwise Φ_{ST} values among Fitzroya cupressoides populations. Dashed lines indicate nonsignificant branches ($P > 0.05$).

Table 5 Shannon's diversity measure values (S) for 12 *Fitzroya cupressoides* populations (A–L) and mean Shannon's measure averaged over all populations (S_{pop}). 95% confidence intervals are given in parentheses

Population	S
A	0.349 (0.110)
B	0.496 (0.112)
C	0.516 (0.115)
D	0.611 (0.100)
E	0.600 (0.102)
F	0.551 (0.100)
G	0.648 (0.098)
H	0.574 (0.105)
I	0.476 (0.108)
J	0.612 (0.100)
K	0.553 (0.103)
L	0.576 (0.104)
Mean, S_{pop}	0.547 (0.045)

This apparent loss of genetic variation has been attributed to a severe reduction in population size during the last glacial period (Fowler & Morris 1977). Low genetic variability recorded in a number of southern temperate conifers has similarly been attributed to the occurrence of range contractions in the past (Hawkins & Sweet 1989; Billington 1991; Shapcott 1991; Gibson *et al*. 1995).

The evidence for a severe range contraction in *Fitzroya* is largely based on results from pollen analysis, together with glaciological evidence. At the peak of the last ice age, an ice sheet covered the Andes, and extended over much of the current land surface of southern Chile below around 43°S, providing little scope for forest survival (Villagrán *et al*. 1995). Further north, in what is today the Chilean Lake District (approximately between 39°S and 43°S), with an ice sheet covering the Andean range, solifluction and glaciofluvial activity probably resulted in the Central Depression and much of the Coastal Range remaining deforested (Villagrán *et al*. 1995). Although the Coastal Range remained largely unglaciated, periglacial activity probably limited the development of forest below altitudes of between 100 m at the southern (Chiloé) and northern (Nahuelbuta) limits of this region (Veit & Garleff 1995). Forest refugia during the last glacial period therefore appear to have been restricted to lower altitudinal areas of the Coastal Range (Cordillera Pelada), the Cordillera Nahuelbuta slightly further north, and the northern Pacific coastal range of Chiloé island (Armesto *et al*. 1995). As a result of greater aridity further north, which limited the potential for northerly migrations, forest refugia west of the Andes were probably limited to latitudes between 36°S and 42°S (Markgraf *et al*. 1995, 1996). Given the limited additional area of land surface exposed by the lower sea levels at that time, the extent of forest areas must have been considerably lower than at present.

Data from pollen analysis further indicate a severe range contraction for *Fitzroya* during glacial times. Between 12 500 and 9500 BP, analysis of the distribution of *Fitzroya*-type pollen within Chile suggests that the species was concentrated in or near the Coastal Range, being most abundant in the north of Chiloé island during this period (Villagrán 1991). Following glacial retreat after 9500 BP, the species appears to have extended its range towards the north and east (Villagrán 1991; Armesto *et al*. 1995).

Evidence therefore suggests that *Fitzroya* may have undergone a severe population bottleneck, perhaps several times during the Pleistocene era as a result of repeated glaciations (Veit & Garleff 1995). However, the results of this investigation suggest that this has not had any appreciable effect on the extent of intraspecific variation, as a significant degree of variation was recorded. Traditionally, assessments of genetic diversity are based on the analysis of allele frequencies, enabling calculation of standard population genetic parameters such as H_S, H_T

and F_{ST} or G_{ST} (Nei 1973; Weir & Cockerham 1984). Although markers possessing codominant inheritance (such as isozymes or microsatellites) provide suitable data for calculating such parameters, those which show dominant inheritance (such as RAPDs) are less amenable to this form of analysis. Although estimates of allele frequency may be obtained from RAPD data by making a series of assumptions and corrections (Lynch & Milligan 1994), this is not possible for *F. cupressoides* as the mating system is not known. Instead, the extent of variation recorded within *Fitzroya* using RAPDs is most readily compared to results from other species by using phenotypic diversity indices such as Shannon's information index (Lewontin 1972), which has been widely used for this purpose (Chalmers *et al*. 1992; Dawson *et al*. 1995; Gillies *et al*. 1997; Wolff *et al*. 1997).

Comparison with Shannon's indices derived for other species is complicated by the fact that other studies have employed a variety of different approaches in its calculation (Isabel *et al*. 1995). Here, we support the approach adopted by Lewontin (1972) and Yeh *et al*. (1995), where Shannon's index is calculated separately for each putative locus, and the mean value of the index is then produced by averaging over all loci. This approach ensures that estimates of phenotypic diversity from each putative locus are equally weighted in the calculation of the S_{pop} diversity statistic. Most other investigations which have used Shannon's index to estimate diversity from RAPD data (Chalmers *et al*. 1992; Dawson *et al*. 1995; Gillies *et al*. 1997; Wolff *et al*. 1997) have employed different methods of calculation, which result in the value being dependent on the sample size, the number of loci scored, or the primers used. Adopting the approach employed by Yeh *et al*. (1995), which avoids these biases, gives a range of values between 0 and 1 (maximum phenotypic diversity). The mean value of S_{pop} of 0.547 recorded here compares with a value of 0.651 for eight *Populus tremuloides* populations surveyed by Yeh *et al*. (1995). Recalculation of the RAPD data presented by Gillies *et al*. (1997) for the tropical broadleaved tree *Cedrela odourata*, following the approach employed by Yeh *et al*. (1995), gives an S_{pop} value of 0.427. These data indicate that the extent of intraspecific variation recorded within *Fitzroya* is therefore comparable with other tree species studied, and provides no evidence for relatively low variability within the species.

Most of the variation in *Fitzroya* (85.6%) was recorded within populations, a result consistent with those from most other woody perennial, outbreeding plant species (Hamrick *et al*. 1992). However, a significant degree of population differentiation was also recorded. The extent of this differentiation is particularly striking, given the restricted geographical range of the species and the fact that the species is wind-pollinated and wind-dispersed. For example, isozyme studies of widely distributed European tree species such as *Fagus sylvatica*, *Pinus sylvestris*, *Quercus petraea* and *Quercus robur* each found that differentiation between populations (G_{ST}) accounted for less than 6% of the total variation recorded (Kinloch *et al*. 1986; Comps *et al*. 1990; Zanetto *et al*. 1994). From a review of 195 isozyme studies of long-lived perennial woody taxa, Hamrick *et al*. (1992) recorded an overall mean G_{ST} value of 8.4%. Although results from RAPD analyses may not be directly comparable to those obtained with isozymes, the results of the current investigation indicate that there is significant population differentiation within *Fitzroya*. A number of studies have recorded a relatively high degree of population differentiation using RAPDs (Gillies *et al*. 1997; Chalmers *et al*. 1992; Moreau *et al*. 1994; Dawson *et al*. 1995), which may result from a contribution of maternally inherited organelle DNA to the RAPD phenotypes observed (Aagaard *et al*. 1995). In the case of *Fitzroya*, where as a member of the Cupressaceae both mitochondrial DNA (mtDNA) and chloroplast (cpDNA) are paternally inherited (Mogensen 1996), this is unlikely to have influenced the results. Inflated estimates of population differentiation using RAPDs may also result from artefacts of statistical analysis (Lynch & Milligan 1994), but the method adopted here (using AMOVA) is considered to produce relatively unbiased estimates (Isabel *et al*. 1995).

Although a degree of overlap was recorded between populations, the results of the PCO analysis highlighted differentiation between three main population groups within *Fitzroya*: the northern Coastal Range (A, B), the southern Coastal Range and Central Depression of Chile (C, D, E, F), and the Andean populations (G, H, I, K, L). Results from the UPGMA dendrogram based on Φ_{ST} values were broadly consistent with this pattern, but emphasized the difference between the northern Coastal Range populations and the others, as well as the distinction between the Argentinian Andean populations and those from Chile. Such differences between populations suggest either that they have been genetically isolated at some time in the past, and/or gene flow between them is restricted at the present time, providing scope for genetic differentiation as a result of drift or selection.

The apparent genetic isolation of the Astillero population as shown in the pairwise Φ_{ST} dendrogram is not clearly evident in the PCO plot where it overlaps with central valley and coastal range populations. No population-specific RAPDs were observed for Astillero, which would clearly demonstrate its genetic isolation. Further investigation of this population is necessary to determine if it is as genetically distinct as this RAPD study suggests.

It is pertinent to consider these results in the context of the postglacial history of the species, as indicated by pollen analysis. If, as pollen evidence suggests, Chilean populations of *Fitzroya* were restricted to coastal refugia during the last glacial period, then populations currently

located in the Central Depression and Andes may have been derived from one or more of these refugia. The closer relationships between populations from the southern Coastal Range and Chiloé and those in the Central Depression and Andes, compared with those of the northern Coastal Range, suggest that the former rather than the latter may have been the main source for postglacial colonization of inland areas. This is entirely consistent with evidence from pollen analysis (Villagrán 1991).

The significant difference recorded between the Argentinian population and those in Chile was an unexpected and interesting result. In Argentina, *Fitzroya* exists in relatively small, scattered populations, mostly at the head of glaciated valleys within a few kilometres of the Chilean border. Although the Andes may be expected to present a significant barrier to gene flow at the present time, they are traversed by a number of forested corridors lying below 1200 m, the current altitudinal limit of *Fitzroya*. The possibility therefore exists that Argentinian populations of *Fitzroya* were derived from those in Chile by postglacial migration. However, such a possibility is not supported by the current data. Although the two Argentinian populations were separated by ≈ 170 km, they were genetically more similar to each other than to any of the Chilean populations, some of which were situated much closer (< 70 km away). These data therefore suggest that the Argentinian populations may be derived from glacial refugia situated east of the Andes. Whether forest refugia were able to persist east of the Andes during the Pleistocene glaciations has been the subject of some debate. Most of the available palynological data suggest that this area remained treeless during glacial periods between the latitudes at which *Fitzroya* now occurs (Markgraf *et al*. 1996), although some recent evidence suggests that local forest refugia may have existed at around 41°S (Bianchi 1997). It has also been suggested that forest taxa spread from a great number of microclimatologically favourable sites where they had survived during full-glacial times (Markgraf *et al*. 1995). The degree of overlap recorded here between Andean populations in both Chile and Argentina may reflect current gene flow between populations which have historically been separated, or could conceivably be a consequence of postglacial colonization of the Chilean Andes from both Chilean and Argentinian refugia. A comparable extent of genetic variation detected by isozymes either side of the Andes, together with the presence of low-frequency alleles in both eastern and western populations of *Fitzroya*, strongly supports the hypothesis of multiple refugia through glacial periods in northern Patagonia (Premoli 1998). In addition, a decrease in the extent of genetic variability in Chilean populations towards the Andes indicated by isozyme analyses suggests migration by long-distance events from coastal glacial refugia (Premoli 1998).

To test such hypotheses, further analyses would be required. Postglacial migration pathways have successfully been investigated in a number of north-temperate tree species using maternally inherited molecular markers, which generally display a higher degree of population differentiation than nuclear markers (Newton *et al*. 1998). For example in European populations of oak (*Quercus* spp.; Ferris *et al*. 1993; Petit *et al*. 1993), beech (*Fagus sylvatica*; Demesure *et al*. 1996) and pine (*Pinus sylvestris*; Sinclair *et al*. 1998), the geographical distribution of maternally inherited haplotypes is consistent with data from pollen analysis indicating a northward migration from different forest refugia in southern Europe following glacial retreat. However, maternally inherited markers are not available in *Fitzroya*, as unusually among higher plants both the mtDNA and cpDNA are thought to be inherited paternally (Mogensen 1996). Studies of molecular phylogeography, which would potentially enable migration routes and the position of putative refugia to be defined more precisely for *Fitzroya*, would therefore require the development of appropriate nuclear markers, perhaps using PCR–RFLP approaches (Newton *et al*. 1998). Genetic evidence from isozyme markers has also been used previously as proxy data to elucidate biogeographical patterns in plants (Wheeler & Guries 1982). Such analyses would be highly instructive, as the pattern of forest refugia and subsequent migration appears to differ very markedly in southern temperate areas compared to the northern temperate regions which have been studied more intensively (Markgraf *et al*. 1995).

The current results have a number of implications for the development of conservation strategies for *Fitzroya*. The detection of population differentiation using techniques such as RAPDs may assist in the definition of appropriate units for conservation (Moritz 1994a, b; Newton *et al*. 1998). For example, it may be inferred that gene flow is likely to be or to have previously been limited between populations which are genetically distinct, indicating some degree of demographic independence (Moritz 1994a). The detection of population differentiation may therefore usefully inform the definition of genetic management units (MUs *sensu* Moritz 1994a; or GRMUs *sensu* Millar & Libby 1991), providing an appropriate focus for conservation management or monitoring (Moritz 1994a).

In *Fitzroya*, the three main population groupings detected within Chile (north Coastal Range, south Coastal Range/Central Depression, Andean) may therefore merit individual conservation attention. The populations in the northern Coastal Range of Chile would appear to be particularly distinctive genetically, and should perhaps be accorded highest priority for conservation. Although substantial populations of *Fitzroya* are protected within National Parks, within Chile these are mostly centred

on the Andes rather than the Coastal Range; the *Fitzroya* populations in the northern Coastal Range are known to have been heavily logged earlier this century (Golte 1996). These results also indicate that the Argentinian populations of *Fitzroya* (most of which are already protected within National Parks) may also be of particular importance for the conservation of genetic diversity within the species. The definition of such management units will also be of value for informing the sampling of germplasm for *ex situ* conservation activities (Allnutt *et al*. 1998), and for sourcing material for restoring degraded populations of the species, an activity which is currently being initiated within Chile.

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