

## ADAPTATION OF MONKEY PUZZLE TO ARID ENVIRONMENTS REFLECTED BY REGIONAL DIFFERENCES IN STABLE CARBON ISOTOPE RATIO AND ALLOCATION TO ROOT BIOMASS

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### ABSTRACT

Glasshouse trials were conducted for nine provenances of monkey puzzle (*Araucaria araucana*), a vulnerable tree native to southern South America that occurs over a wide ecological range (annual precipitation ranges from the western to the eastern side of the Andean Range from more than 4000 to less than 200 mm, and elevation ranges from 600 to almost 2000 m above sea level). Samples of needle tissues were used to determine stable carbon isotope  $\delta^{13}\text{C}$  values, a time-averaged indicator of water use efficiency. A significant relationship was found between  $\delta^{13}\text{C}$  and mean annual rainfall with higher (less negative)  $\delta^{13}\text{C}$  ratios found for populations within the drier Argentinian region. Root mass ratio was also found to be significantly correlated with mean annual rainfall, providing further evidence for genetic adaptation to drought. This study demonstrates the utility of carbon isotope discrimination in describing genetic adaptation to arid environments, although it is probably most useful in detecting differentiation when the strategy of the species under investigation is to increase water use efficiency, rather than drought-avoidance. The results suggest that populations on the eastern and western sides of the Andes should be treated as separate management units for the purposes of conserving the genetic resource of this species.

**Key-words:** *Araucaria araucana*, conservation genetics, carbon isotopes, root mass ratio, water-use efficiency, Patagonia

### INTRODUCTION

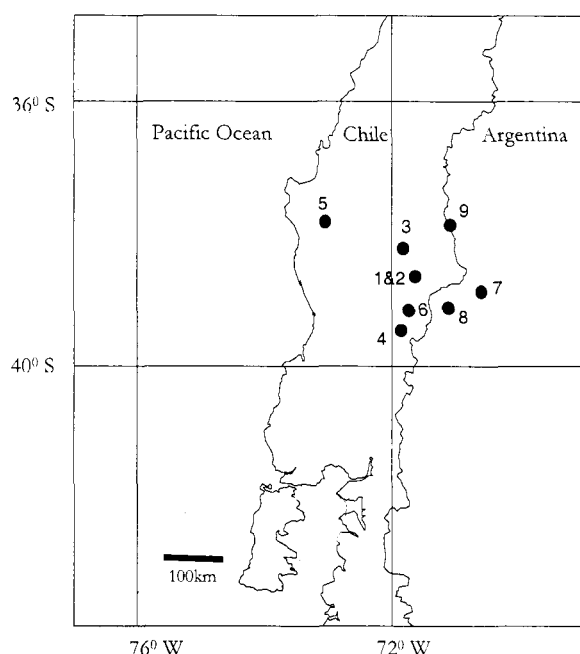
Surveys of genetic variation are often an important facet of endangered species management, with the common objective of preserving genetic adaptability (BURGMAN *et al.* 1993; HOLSINGER & GOTTLIEB 1991). The measurement of quantitative trait variation is particularly relevant as it provides a direct measure of additive genetic variability, and hence the adaptive potential of populations for ecologically relevant traits (ENNOS 1996; LANDE & BARROWCLOUGH 1987). This approach provides data on levels of genetic variation within populations and adaptive differences among populations, allowing evaluation of the effects of reduced variability on fitness traits (STORFER 1996).

Tolerance to drought is often used to determine the suitability of provenances for tree breeding and refore-

tation programs (DONOVAN & EHLERINGER 1994; GRIFFITHS 1993; KAVANAGH *et al.* 1999; NEWTON *et al.* 1996; ZHANG *et al.* 1994). One component of drought tolerance is water-use efficiency (WUE) which can be assessed by stable carbon isotope discrimination ( $\delta^{13}\text{C}$ ), providing a time-integrated measure (FARQUHAR *et al.* 1988; FARQUHAR & RICHARDS 1984). In general, plants that are better adapted to low rainfall and drought show enrichment in their bulk  $\delta^{13}\text{C}$  values (HUC *et al.* 1994). Several studies have established that  $\delta^{13}\text{C}$  is a largely heritable trait, subjected to close genetic control (HUBICK *et al.* 1988; Farquhar *et al.* 1989; MARTIN & THORSTENSON 1988). Furthermore,  $\delta^{13}\text{C}$  has been related to variation in plant growth characteristics (EHLERINGER 1993; NGUGI *et al.* 1994; MENCUCINI & COMSTOCK 1999; ZHANG *et al.* 1997) with significant relationships found with characteristics such as hydrau-

**Table 1.** Name, location, mean annual rainfall and altitude of nine Monkey Puzzle populations sampled.

Site Name and Population Identifier	Region	Latitude (S)	Longitude (W)	Mean Annual Rainfall (mm)	Altitude (m)
1 Conguillío (low altitude)	Chilean Andes	38°39'02"	71°37'18"	1197	1080
2 Conguillío (high altitude)	Chilean Andes	38°39'02"	71°37'18"	1197	1570–1645
3 Tolhuaca	Chilean Andes	38°12'93"	71°48'46"	1653	1080
4 Vilarrica-Palguin	Chilean Andes	39°27'53"	71°51'15"	2351	1215–1335
5 Nahuelbuta	Coastal Chilean	37°49'38"	73°02'03"	1055	1400
6 Huerquehue	Chilean Andes	39°09'40"	71°42'97"	2543	1210–1255
7 Primeros Pinos	Argentinian Andes	38°52'51"	70°35'10"	357	1670
8 Largo Pulmari	Argentinian Andes	39°07'15"	71°06'06"	173	1025
9 Caviahue	Argentinian Andes	37°51'70"	71°04'19"	241	1030

**Figure 1.** Sketch map showing the position of Monkey Puzzle populations sampled during this investigation (1–9; see Table 1 for details).

lic architecture and leaf size, seedling height and grain yield. However, few attempts have been made to describe genetic variation in WUE within forest tree species using carbon isotope discrimination (eg. ANDERSON *et al.* 1996; ZHANG *et al.* 1993; ZHANG & MARSHALL 1995).

The species under investigation in the present study, *Araucaria araucana* (Molina) K. Koch (Araucariaceae), commonly known as the Monkey Puzzle tree or Pehuén, is a vulnerable tree of exceptional ecological, economic and cultural significance, endemic to southern Chile and Argentina. Monkey Puzzle is an impressively large and long-lived conifer, attaining 50 m in height, 2.5 m in girth and reaching ages of at least 1300 years (MONTAL-

DO 1974) and is of conservation concern, owing to extensive historical clearance and current human-induced pressures.

Although Monkey Puzzle has a very restricted natural distribution, it occurs over a wide ecological range (VEBLEN 1982). Annual precipitation decreases across the Andes from more than 4000 mm on west-facing slopes in Chile to less than 200 mm on the Argentinean steppe, over an elevation range from 600 to almost 2000 m above sea level (DELMASTRO & DONOSO 1980). In view of this, together with the geographic separation of coastal and Andean populations, some degree of genetic differentiation may be expected. DELMASTRO & DONOSO (1980) speculated that racial divergence was probable between coastal and Andean populations and observed some differences in plant form and the degree of vegetative reproduction, although the genetic basis of these traits was not assessed. They also predicted differences across the Andean range, as a result of the major climatic variation.

Despite its importance, very little information is available concerning patterns of genetic variation in this species. In a previous study, RAFII & DODD (1998) investigated the genetic diversity of Monkey Puzzle using proportional composition of foliar epicuticular wax alkanes; a method that is likely to detect differences produced by the distinct growing environments on the eastern compared to the western side of the Andes. This study revealed a high level of intrapopulation variation, as well as a regional difference between the eastern and western sides of the Andes. This was thought to reflect genetic adaptation to the more arid conditions on the eastern side of the Andes. However, only a small number of populations (4) were sampled in this analysis, from a very restricted geographical area.

The present study investigates the quantitative trait variation within and among nine populations of Mon-

key Puzzle collected as seed from throughout the natural range and grown in a glasshouse. The experiment formed part of a larger study that included analysis of neutral DNA marker variation to develop comprehensive recommendations concerning the management of the genetic resource of this species. Well-watered conditions were maintained, as  $\delta^{13}\text{C}$  measured in a common environment with ample resources is the best measure of the genetic or adaptive set point for WUE (CONDON & RICHARDS 1992). The primary objective was to test for genetic variation in intrinsic WUE as assessed by  $\delta^{13}\text{C}$ , and to determine whether such variation was correlated with geographic variation in water availability or altitude. Variation in root mass ratio was also examined as an additional quantitative trait related to drought adaptation.

## MATERIALS AND METHODS

### Study Sites and Sample Collection

1107 seeds were collected from a total of 181 trees within nine populations located throughout the natural range of Monkey Puzzle (Table 1 and Fig. 1). The sites were chosen to represent nearly the entire distribution of the species, including areas on both Chilean and Argentinian sides of the Andes and within the Coastal Range of Chile. Annual rainfall statistics were obtained for each site from the website of the NATIONAL CLIMATE DATA CENTRE (2000). Altitude was obtained at each site using a digital altimeter.

Trees were selected randomly for sampling within each population, but were separated by a minimum distance of 100 m where possible to avoid sampling closely related or genetically identical individuals (vegetative propagation of Monkey Puzzle has been reported and observed in a number of natural stands (SCHILLING & DONOSO 1976)). As many seeds as possible (up to 25) were collected from underneath 20 trees per population and were stored in plastic bags for a period of 3 weeks.

Seeds were sown into 1452 cm<sup>3</sup> polyethylene pots containing potting mix with peat, mixed at a ratio of one to three by volume. Seedlings of Monkey Puzzle were raised and established in a fully randomised design in a glasshouse at the Royal Botanic Gardens Edinburgh, UK. The seedlings were placed on a heated bench top (16 to 18 °C) at a height of 1 m. The bench top had a plastic seal to prevent rooting into the gravel outside the pots. Seedlings were grown for a period of 21 months (May 1999 to February 2001) under ambient light conditions in ventilated and temperature controlled conditions (temperature maintained between 7–26 °C).

Seedlings were watered every 2–3 days to keep the soil moist at all times and drenched once with pesticide (Intercept) in May 2000.

### Carbon isotope determination: analytical procedure

Seedlings were chosen for measurement rather than adult trees due to the extremely slow growth rate of the species and because seedlings are likely to be the most drought sensitive stage of the life cycle. After 13 months of growth, needle tissue was harvested from seven to 10 plants from each of the nine populations (86 seedlings in total), ensuring needles were healthy, from the most recent growth flush that had developed under completely unshaded conditions. Each plant chosen for sampling originated from a different maternal tree. This restricted the number of samples for some small populations, as few trees were producing seed. The samples were oven dried at 70 °C for 48 hours. After drying, the samples were ground to a fine homogeneous powder using a liquid nitrogen cooled mill (SPEX 6700 freezer mill, Glen Creston Ltd., Stanmore, Middlesex, UK). The  $^{13}\text{C}/^{12}\text{C}$  ratio of this material was measured (Natural Environment Research Council <sup>15</sup>N Stable Isotope Facility, CEH Merlewood) and an average and standard deviation calculated from the replicates of each population. 1mg of each ground sample was weighed using a high precision microbalance (Sartorius Ltd) and then sealed into a 6 × 4 mm tin capsule. Samples were then combusted using an automated Carlo Erba NA1500 elemental analyser coupled to a Dennis Leigh Technology Isotope Ratio Mass-Spectrometer. A working standard of beet sucrose was analysed after every tenth sample resulting in an analytical precision of 0.15 ‰. Results are expressed in  $\delta$  notation; i.e.  $\delta^{13}\text{C} = [(R_{\text{sample}} / R_{\text{standard}}) - 1] \times 1000$  (‰) where R is the ratio of  $^{13}\text{C}$  to  $^{12}\text{C}$  in the sample and standard accordingly. All  $\delta^{13}\text{C}$  results are expressed relative to the international standard Pee Dee Belemnite.

Samples were loaded into the autosampler and each in turn was dropped into a 1000°C combustion furnace coinciding with a pulse of O<sub>2</sub>. The carrier gas carries the gaseous combustion products into a 600°C reduction furnace packed with copper wires. This removes any unused O<sub>2</sub> from the combustion and reduces oxides of N back to N<sub>2</sub>. After passing through a Mg(ClO<sub>4</sub>)<sub>2</sub> water trap and a packed GC column, a small proportion of carrier gas containing sample CO<sub>2</sub> is diverted into the inlet of the mass spectrometer. The mass spectrometer determines the ratio of  $m/z$  44, 45 and 46 and compares these to the corresponding ratios in a standard of known  $\delta^{13}\text{C}$ .

### Root Mass Ratio

Root and shoot mass were measured after a 21-month period of growth. Between eight to eleven seedlings from each population (89 seedlings in total) were harvested and roots were separated at ground level and washed. Each plant chosen for sampling originated from a different maternal tree. Roots and shoots were oven dried separately in paper bags for 48 hours at 80 °C and then weighed. Root mass ratio was calculated as the ratio of root dry mass to total dry mass.

### Data Analysis

Regression analysis was conducted between mean population values for  $\delta^{13}\text{C}$ , root mass ratio and mean annual rainfall, altitude and latitude (MINITAB 1999). Transformations were used to correct for non-linearity where visual inspection revealed non-linear patterns. The critical value of  $P < 0.05$  was used to determine the significance of the differences recorded. Power calculations were performed on non-significant results using GPOWER (FAUL & ERDFELDER 1992).

Additionally, root mass ratio and  $\delta^{13}\text{C}$  were evaluated by analysis of variance (ANOVA) (MINITAB 1999). Variance components were estimated for (i) within populations, (ii) among populations, and (iii) among the regions, where regions are defined as populations within Argentina (east side of the Andes), populations on the Chilean side of the Andes and populations in the Chilean Coastal Range. This stratification is based on predictions of regional genetic divergence DELMASTRO & DONOSO (1980) and reflects broad suites of coincident environmental attributes and regional classifications made in other studies (BEKESSY *et al.* in press).

## RESULTS

### Carbon-isotope discrimination

Of the environmental parameters considered, mean

annual rainfall was the only factor significantly correlated with  $\delta^{13}\text{C}$  ( $P < 0.05$ ) (Table 2, Fig. 2(a)), with a logarithmic regression providing the best fit ( $r^2 = 0.567$ ). The relationship between  $\delta^{13}\text{C}$  and altitude in the present study was not found to be significant, despite the wide range of altitudes sampled (1080 to 1670 m). However, the measured correlation was 0.35 and the power of the test (b) was only 0.26 at the a level of 0.05. That is, there was only a one in four chance of finding a statistically significant association, even if the correlation of 0.35 was biologically caused. Thus, the test was not adequate to demonstrate unequivocally that the trend was absent.

The overall variation in  $\delta^{13}\text{C}$  was relatively high, particularly given the well-watered conditions under which seedlings were grown, ranging from  $-29.78$  to  $-24.80$  ‰ (typical values for  $\text{C}_3$  plants range from  $-20$  to  $-35$  ‰ (JONES 1992)). Mean  $\delta^{13}\text{C}$  values did not differ significantly among populations (Table 3) and differences among populations did not account for a substantial component of the variation (although the power of the test was not strong,  $b = 0.29$  at  $a = 0.05$ ). However, region was found to be significant by ANOVA ( $P < 0.025$ ), explaining 12% of the total variation. Mean values for regions (within Argentina (east side of the Andes), on the Chilean side of the Andes and in the Chilean Coastal Range) were  $-26.92$ ,  $-27.39$  and  $-27.16$  ‰ respectively.

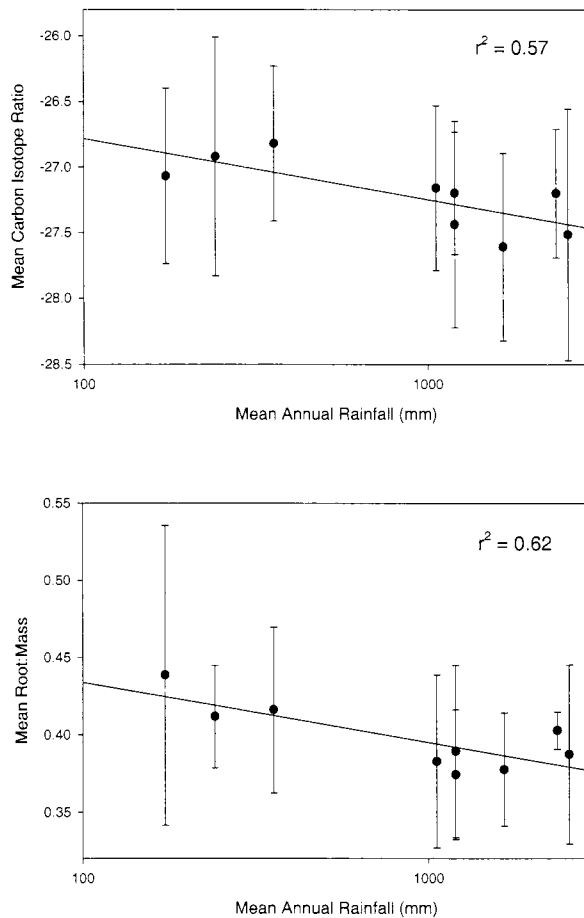
### Root Mass Ratio

The regression between root mass ratio and mean annual rainfall was also significant ( $r^2 = 0.624$ ;  $P < 0.05$ ) with a logarithmic regression providing the best fit (Table 2, Fig. 2(b)). Among population variation was not found to be significant by ANOVA (Table 3) ( $b = 0.29$  at  $a = 0.05$ ). However, root mass ratio was found to vary significantly among regions ( $P < 0.025$ ), explaining 13.5 % of the total variation. Mean root mass ratios for regions (within Argentina (east side of the Andes), on the Chilean side of the Andes and in the Chilean Coastal Range) were 0.42, 0.38 and 0.38

Table 2. Regressions ( $r^2$ ) between population means of  $\delta^{13}\text{C}$  and root mass ratio on environmental parameters. All regressions were linear except those indicated.

	Mean Annual Rainfall	Altitude	Latitude	$\delta^{13}\text{C}$	Root:Mass
Mean Annual Rainfall	-				
Altitude	0.001	-			
Latitude	0.162	0.018	-		
$\delta^{13}\text{C}$	0.567(log)*	0.124	0.005	-	
Root:Mass	0.624(log)*	0.003	0.108	0.532*	-

\* $P < 0.05$



**Figure 2.** Logarithmic regression of mean annual rainfall (mm) against (a) mean  $\delta^{13}\text{C}$  ( $P = 0.019$ ) and (b) mean root mass ratio ( $P = 0.011$ ) for 9 monkey puzzle populations (error bars shown).

respectively. The regression of root mass ratio on  $\delta^{13}\text{C}$  was significant ( $r^2 = 0.532$ ;  $P < 0.05$ ) (Table 3).

**DISCUSSION**

This study found that Monkey Puzzle seedlings from drier habitats produce higher (less negative) values of carbon isotope discrimination and have greater allocation to root mass relative to total mass. The significant, although weak, regression between both  $\delta^{13}\text{C}$  and root mass ratio with mean annual rainfall suggests that adaptive variation is related to the extreme rainfall gradient that exists across the Andean range. In addition to the regional difference detected by ANOVA, these results suggest that plants originating from the more arid Argentinian region are able to achieve higher water use efficiency than those from the more mesic Chilean region under equivalent conditions. Additionally plants from Argentina demonstrate greater allocation to root mass, which is an advantage for survival in arid environments, providing more effective water uptake (JONES 1992). These results are consistent with RAFII & DODD (1998) who also found differentiation between populations grown east compared with west side of the Andes, when comparing the proportional composition of foliar epicuticular wax alkanes.

The wide range of values of carbon isotope discrimination for populations of Monkey Puzzle ( $-24.8\%$  to  $-29.78\%$ ) provides an indication of high levels of genetic variation. BRODRIBB & HILL (1998) studied the photosynthetic drought physiology of 13 other Southern Hemisphere conifers and used their results to make generalisations about the distribution of conifer species; genera shown to be most drought tolerant were more widespread than the most drought sensitive genera. The wide range of values for Monkey Puzzle suggests that it is capable of existing in greatly varying rainfall environments. This provides an interesting deviation to the theory that current climatic conditions are partly driving the gradual replacement of Monkey Puzzle by

**Table 3.** Analysis of variance of  $\delta^{13}\text{C}$  and root mass ratio for nine Monkey Puzzle populations from the Argentinian Andes, Chilean Andes and Coastal Chilean regions.

		d.f.*	MSD+	Variance Component	% of total variance	Pvalue*
$\delta^{13}\text{C}$	Among regions	2	1.9806	0.07	12.22	<0.025
	Among populations within regions	6	0.2702	-0.03	-0.00	-
	Within populations	77	0.5086	0.51	87.78	-
Root:Mass	Among regions	2	0.0121	0.00	13.54	<0.025
	Among populations within regions	6	0.0014	0.00	0.00	-
	Within regions	80	0.0027	0.00	86.46	-

\*Degrees of freedom  
 +Mean squared deviations  
 +Significance of the variance components

angiosperm tree species (SCHMITHUSEN 1960). As a member of a genus that may have originated as early as the Jurassic period (STOCKEY 1990), it seems likely that adaptation to a broad environmental range has probably been critical to the species long-term survival.

The relationship found in this study between carbon isotope discrimination and mean annual rainfall is consistent with current theory (FARQUHAR *et al.* 1988; FARQUHAR & RICHARDS 1984) and with several other studies on forest tree species (ANDERSON *et al.* 1996; DONOVAN EHLERINGER 1994; NEWTON *et al.* 1996). However, the relationship between  $\delta^{13}\text{C}$  and rainfall does not seem to be unequivocal for all species in all circumstances, and conflicting results can be found in the literature. For example BRODRIBB & HILL (1998) found no consistent trend of  $\delta^{13}\text{C}$  with aridity. Likewise, ZHANG *et al.* (1993) failed to find higher WUE in Douglas-fir from drier habitats and both MILLER *et al.* (2001) and SCHULZE *et al.* (1991) found no consistent trend of  $\delta^{13}\text{C}$  along a rainfall gradient for a range of tree species. Estimation of statistical power is essential to determine whether negative results are conclusive (AKÇAKAYA *et al.* 1997). Additionally, there is a need to identify and be aware of possible confounding effects.

One confounding effect may stem from whether plants are grown in a controlled environment or are measured *in situ* in contrasting rainfall environments, where other factors (e.g., temperature, nutrient levels) may co-vary with variations in rainfall. Additionally, stomatal conductance has been found to be a highly plastic character (WOODWARD 1986), so differences measured *in situ* may not be genetically controlled. Populations measured *in situ* are generally found to have more significant and consistent relationships with environmental factors, such as altitude and rainfall (for Example, Cordell *et al.* 1998; NEWTON *et al.* 1996; NGUGI *et al.* 1994). Furthermore, plants grown in a controlled environment, but under water-stressed growing conditions generally demonstrate greater variation in  $\delta^{13}\text{C}$  than plants grown in well-watered conditions (for example, DONOVAN & EHLERINGER 1994; LI *et al.* 2000). Differences in the tissue chosen for analysis may also affect results in studies of carbon isotope discrimination (ZHANG & CREGG 1996; MILLER *et al.* 2001)

In addition, inconsistencies in  $\delta^{13}\text{C}$  studies may be explained by the different survival strategies adopted by drought-affected species; whether it be to increase water use efficiency, or drought-avoidance by stomatal closure (using the classification system of JONES (1992)). For example, conifers within the genera *Callitris* have been found to produce relatively indiscriminate water use while water is available, but reduce

stomatal conductance to very low levels as soon as soil water potential drops (ATTWILL & CLAYTON-GREENE 1984). ZHANG *et al.* (1997) found a similar effect for ponderosa pine. Of course, not all drought-adapted plants will be characterised by modifications to diffusion at the leaf/atmosphere interface. Drought adaptation can be achieved by many other means, including morphological features such as less allocation to leaf area and trunk height and more to root length and depth (MILLER *et al.* 2001). Carbon isotope discrimination may not be an appropriate technique for detecting adaptive genetic variability across a precipitation gradient for species that are drought adapted in these ways.

A correlation between carbon isotope discrimination and altitude of origin has been found in several previous studies (for example, MARSHALL & ZHANG 1994; READ & FARQUHAR 1991), although conflicting trends have been demonstrated, suggesting that altitude may be a secondary effect (BRODRIBB & HILL 1998). The relationship between  $\delta^{13}\text{C}$  and altitude in the present study was not found to be significant, despite the wide range of altitudes sampled (1080 to 1670 m), although the power of the test was low.

It should be emphasised that reciprocal transplant experiments are needed to fully understand patterns of adaptive differentiation (ENNOS 1996). However, on the basis of results from the present study, it is recommended that germplasm from different populations should be separately collected and propagated and that reforestation projects should ensure that local seed is used wherever possible. In particular, germplasm should not be mixed between populations east and west of the Andean range, as it has been shown that these populations are adapted to the contrasting rainfall environments in these regions.

This study is one of very few investigations of quantitative genetic variation in  $\delta^{13}\text{C}$  for forest trees. We have demonstrated that carbon isotope discrimination might be a useful tool for selecting suitable provenances for reforestation projects, although it is probably most useful in detecting adaptation to arid environments when the strategy of the species under investigation is to increase water use efficiency, rather than to avoid droughting. Experimental design is also critical, as measurements must be taken on plants grown in a controlled environment to ensure that the variation represents genetic effects, rather than detecting the plastic response to changing environmental conditions across sites. Estimation of statistical power is essential in studies such as this in order to determine whether non-significant responses are conclusive and can lead to meaningful generalisations.

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