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PART OF A HIGHLIGHT ON TREE BIOLOGY

Multi-stemmed trees of *Nothofagus pumilio* second-growth forest in Patagonia are formed by highly related individuals

Irène Till-Bottraud[1,*], Alex Fajardo[2] and Delphine Rioux[1]

[1]*Laboratoire d'Ecologie Alpine, UMR CNRS 5553, Université Joseph Fourier-Grenoble 1, BP 53, F–38041 Grenoble Cedex 9, France and* [2]*Centro de Investigación en Ecosistemas de la Patagonia (CIEP) Conicyt-Regional R10C1003, Universidad Austral de Chile, Ignacio Serrano 509, Coyhaique, Chile*
** For correspondence. E-mail irene.till@ujf-grenoble.fr*

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• *Background and Aims* Multi-stemmed trees (tree clusters) in *Nothofagus pumilio*, a dominant tree species in Patagonia, are very uncommon and are restricted to the edge of second-growth forests following human-provoked fires. No vegetative reproduction has been reported so far. The genetic structure of multi-stemmed trees of this species was investigated and it was hypothesized that genets within a cluster were more closely related than average in the population.
• *Methods* Fifteen clusters (composed of at least three purported stems) and 15 single trees were sampled at the edge of a second-growth forest and genotyped using two amplified fragment length polymorphism (AFLP) primer pairs. We obtained 119 polymorphic markers that allowed clonality to be determined, together with sibship structure and relatedness among samples.
• *Key Results* Clonality was detected in seven clusters but all clusters had at least two different genotypes. Full sibs were found exclusively within clusters and in all clusters. Within a cluster, stems that were not identified as full sibs were often half sibs. Relatedness values for the full sibs and half sibs were higher than the theoretical values of 0·5 and 0·25 but the relatedness between clusters was very low.
• *Conclusions* Tree clusters that are merged at the edge of the second-growth forest of *N. pumilio* are composed of stems of the same genotype and of other genotypes that are highly related (but not always). It is suggested that this peculiar genetic structure results from a combination of several causes, including selection for merging of related individuals.

Key words: AFLP, multi-stemmed trees, Patagonia, post-fire regeneration, sibship structure.

INTRODUCTION

In some tree species, trees grow in merged clusters, forming multi-stemmed trees. Most often, this is due to several shoots growing from the same root system, either naturally, or, more frequently, as a consequence of sprouting after major disturbance events (fires, droughts, hurricanes; Bond and Midgley, 2001; Del Tredici, 2001). This pattern can also be observed at the upper forest edge in mountains or at the northern limit of arctic forests where trees lose their apical dominance and grow in creeping or multi-stemmed growth forms due to the combined effect of snow, wind and low temperatures ('krummholz'; Devi *et al.*, 2008; Wardle, 2008). In all these cases, the different stems are ramets of a single genet resulting from clonal growth. In other cases, however, multi-stemmed trees result from the merging of different individuals as is the case in some pine species of the subgenus *Strobus*. In limber pine (*Pinus flexilis*) (Linhart and Tomback, 1985; Schuster and Mitton, 1991; Carsey and Tomback, 1994), whitebark pine (*P. albicaulis*) (Linhart and Tomback, 1985; Furnier *et al.*, 1987) and Swiss stone pine (*P. cembra*) (Tomback *et al.*, 1993), the merged stems correspond to different individuals, which are on average more related to each other than random individuals from the population. Over an entire stand of *P. albicaulis*, however, individuals in neighbouring clusters are not more similar than individuals in distant clusters (Furnier *et al.*, 1987); consequently, the distribution of genotypes across the population is likely to be random (Jorgensen and Hamrick, 1997). This is contrary to species with wind-dispersed seeds, which typically display associations between the degree of relatedness and distance from a seed source (Hamrick and Nason, 1996). The seeds of all these pine species are dispersed specifically by Clark's (*Nucifraga columbiana*) and spotted (*Nucifraga caryocatactes*) nutcrackers (for *P. albicaulis* and *P. flexilis* or *P. cembra*, respectively), that bury seeds in caches for winter provision. More than 80 % of the seeds are retrieved, but the few remaining seeds germinate and grow into clusters of merged trees. The high relatedness in these clusters indicates that several seeds in a cache are related as half to full siblings (Carsey and Tomback, 1994). Merging was also found in six species of strangler fig trees resulting in single-stem hollow trees (Thomson *et al.*, 1991) with branches differing by only a few loci (one to four isozyme loci out of 18 systems). For tree species with wind-dispersed seeds the genetic composition of merged tree clusters and the relatedness among the genets is largely unknown.

Strong positive interactions among individuals, such as merging, can be interpreted as a form of cooperation. In strangler figs, for example, the benefit for young seedlings to fuse is a gain

in mechanical stability, the hastening of the host's death and the reception of more light and soil resources (Thomson *et al.*, 1991). One of the theories explaining the evolution of co-operation is kin selection (i.e. processes by which traits are favoured because of their beneficial effect on the fitness of relatives; West *et al.*, 2007). Indeed, in *Botryllus schlosseri*, a colonial marine ascidia where survival and onset of reproduction are size-dependent, Grosberg and Quinn (1986) observed a non-random association of relatives with merging of different genotypes occurring only among closely related individuals. Biernaskie (2011) found reduced competitiveness among kin compared with non-kin in *Ipomoea hederacea*. The kin structure of multi-stemmed trees is thus important to assess.

Genetic markers (isozymes, microsatellites, RAPDs, AFLPs) are commonly used to determine the clonal structure of a population (e.g. Escaravage *et al.*, 1998; Galeuchet *et al.*, 2002) and can be used to determine whether multi-stemmed trees are the result of sprouting or merging, i.e. whether the trunks are ramets of the same genet (*sensu* Harper, 1977) or different genets. Identical multilocus genotypes (or almost identical multilocus genotypes in the case of AFLPs to account for somatic mutations and typing errors; Escaravage *et al.*, 1998; Bonin *et al.*, 2004) are considered ramets of a single genet (multilocus lineages; Arnaud-Haond *et al.*, 2007). However, the reliability of the conclusion depends on the variability of the markers: too little variability (low number of polymorphic markers and low number of alleles, as is sometimes the case for allozymes) overestimates the size of genets (Linhart and Tomback, 1985; Galeuchet *et al.*, 2002). Genetic markers also allow estimating the degree of relatedness between individuals. One can either determine sibship structure in a population (i.e. whether two individuals are full sibs, half sibs or unrelated), or compute kinship or relationship coefficients between pairs of individuals (Jones *et al.*, 2010).

Nothofagus pumilio is a dominant tree species of the southern Andes forests of Chile and Argentina (Fajardo and de Graaf, 2004). Unlike most of other *Nothofagus* species, *N. pumilio* has no published occurrence of vegetative sprouting, although at the alpine treeline, Barrera *et al.* (2000) described a krummholz structure with adventitious rooting and consequent vegetative multiplication. However, at the outermost edge of second-growth forests of human-provoked fire origin, the occurrence of multi-stemmed mature individuals seems to prevail (Fajardo and McIntire, 2010; Fig. 1). Fire is not common in the dynamics of this species in central Patagonia and may explain the peculiarity of this phenomenon. Cross-sections through the root collar of these multi-stemmed trees showed that they were merged clusters of separate individuals (i.e. they were found to have multiple origins; Fajardo and McIntire, 2010). All trees originated at approximately the same time (about 45 years ago in the study location of Fajardo and McIntire, 2010) indicating that regeneration occurred as one pulse following fire disturbance. At the edge locations, Fajardo and McIntire (2010) found significantly more merged trees per hectare (1873 ± 379) than in interior second-growth (88 ± 88) and mature stands (zero multi-stemmed trees). They also found that at edge locations seedlings growing in clusters survived significantly better than single planted seedlings, whereas individual seedlings survived significantly better within the forest (Fajardo and McIntire, 2011). The authors explain this as a mechanical facilitation effect against stressful conditions in open areas in Patagonia where winds can be extremely strong. Moreover, merged multi-stemmed trees survive and grow better than single-stemmed trees (McIntire and Fajardo, 2011). Preliminary genetic analyses (dendrogram of genetic distances) indicate that the multi-stemmed trees are composed of different genets (McIntire and Fajardo, 2011). If merging is the result of kin selection for better resource acquisition or better structural stability against wind, these different genets should be genetically related.

In this study we tested the hypothesis that genets within a cluster (a multi-stemmed tree) are more closely related than random individuals from the population. We therefore estimated the sibship structure and the degree of relatedness of

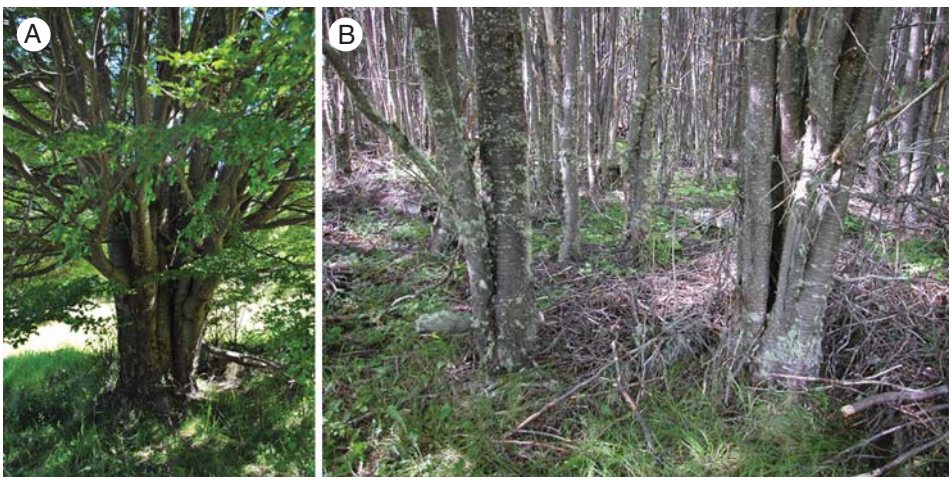

FIG 1. Two photographs taken from the same spot showing: (A) a multi-stem tree located at the very edge of a second-growth forest of *Nothofagus pumilio* in Reserva Coyhaique, Patagonia, Chile (picture taken from inside looking outside the stand); and (B) multi-stem trees intermingled with single-stemmed trees inside the stand (10 m from the edge). In (A) note the many stems forming the multi-stemmed tree and how some of these stems are simultaneously in several phases of merging.

tree samples in an edge of a second-growth forest population of *N. pumilio* using AFLP fingerprinting. We previously needed to (*a*) check whether our dataset was variable enough to address these points and (*b*) confirm that the stems that form a cluster belong to different genets. We then discuss the possible origin of this very peculiar structure in relation to seed dispersal and selection.

MATERIALS AND METHODS

Species description and study site

Nothofagus pumilio (Poeppig et Endlicher) Krasser (lenga, Nothofagaceae) is a wind-pollinated, deciduous, monoecious, shade-intolerant species (Veblen *et al.*, 1996) that exhibits mast seeding at intervals of 3–8 years (Mascareño, 1987). Each deciduous fruit produces at most three seeds that are dispersed principally by gravity and wind (Veblen *et al.*, 1996). The species has thin-barked stems that make it very susceptible to being killed by severe fires (Fajardo and González, 2009). Vegetative reproduction of any kind has not been previously documented or determined for *N. pumilio*, although Veblen *et al.* (1996) and Barrera *et al.* (2000) described the production of adventitious roots following wind-blown trees and in krummholz at the alpine treeline, respectively. Forests of *N. pumilio* in Patagonia are mostly monospecific and represent a transition between the rain temperate forest of western Patagonia and steppe formations at eastern Patagonia (Fajardo and de Graaf, 2004). Thus, open growing (i.e. away from or at the edge of closed canopy) tree seedlings of second-growth forests are likely to be at or approaching an abiotic stress due to desiccation, exacerbated by Patagonian winds.

The study site is located in a second-growth forest of fire origin of *Nothofagus pumilio* in Reserva Coyhaique (45°52′S, 72°00′W, 800 m a.s.l. on average), Coyhaique province, Aysén Region, Chile. The annual precipitation here is approx. 1350 mm (Informe Meteorológico de Chile, Dirección General de Aguas in 2008). The soil is derived from aeolian volcanic ash deposits. The aspect of this second-growth forest is generally south with a slope of $<5°$. In this particular reserve, a large-scale, human-induced fire happened in about 1950 and burnt down some 600 ha of *N. pumilio* old-growth forest. A fire boundary was then created, from where the unaffected old-growth forest of *N. pumilio* spread seeds down slope. Thus, a fringe of regeneration was formed that led, within decades, to a second-growth forest. This sequence of recolonization towards the formation of a second-growth forest in *N. pumilio* is pervasive around this region. Cattle and guanaco (*Lama guanicoe*) commonly browse regeneration of *N. pumilio* (Cavieres and Fajardo, 2005); however, this is not a problem in Reserva Coyhaique, where cattle have been fenced off for decades, and the presence of guanaco in this part of Patagonia is nil.

Sampling

Along the edge (a rather straight band of approx. 150 m) of a second-growth forest of fire origin, we sampled foliage from a selection of 30 adult trees (approx. 45 years) 15 of which were multi-stemmed and 15 single-stemmed. All trees at the immediate edge were multi-stemmed. The selected 30 adult (multi- and single-stemmed) trees were located at closest pair-distances of 3–15 m. As the edge of the second-growth forest is not a sharp line, multi- and single-stemmed trees grow together over a width of 10–15 m (see Fig. 1B). We used random numbers representing the sequence of the trees (e.g. 1st, 2nd … 5th) to select the multi-stemmed trees. Single-stemmed trees (located 10–15 m inside the forest, perpendicular to the edge) were also randomly selected for tissue collection. We collected foliar tissue from three to six stems (i.e. three to six vertical stems at 2 m above ground level) at each merged cluster (Nmerged = 66, Ntotal = 81); the number of stems mostly represented the total amount of stems in a cluster (we sampled a maximum of six stems per cluster). Stem distances within (0·3–1 m) and among multi-stemmed trees (2–6 m) are in the range of other neighbour stands' tree distances (Fajardo and McIntire, 2010). Leaves were placed in labelled plastic bags with silica gel for DNA preservation.

AFLP procedure

Total DNA was extracted using the DNeasy 96 plant extraction kit (QIAGEN) according to the manufacturer instructions. The AFLP procedure, modified from Vos *et al.* (1995), was as follows. Digestion of genomic DNA was performed together with ligation of double stranded adaptors for 2 h in a 11-μL mix using 1 U of *Mse*I, 5 U of *Eco*RI (New England Biolabs) and 1 U of T4 DNA Ligase (Roche). Products were diluted 1 : 10 and pre-selective polymerase chain reaction (PCR; 120 s at 72 °C, 30 cycles at 30 s at 94 °C, 30 s at 56 °C, 120 s at 72 °C, with a final elongation of 10 min at 72 °C) was carried out in a 25-μL volume containing 2·5 μL of Tris–HCl, 1·5 μL of $MgCl_2$ (final concentration 1·5 mM), 2 μL of dNTP mix (final concentration 0·2 mM each), 0·5 μL of each primer (adaptors + one selective base) at 10 μM, and 0·5 U of Ampli*Taq* DNA polymerase (Applied Biosystems). After a 1 : 20 dilution of pre-selective PCR products, the selective amplification (10 min at 95 °C, 13 cycles at 30 s at 94 °C, 60 s at 65 °C to 55 °C, and 60 s at 72 °C, 23 cycles at 30 s at 94 °C, 60 s at 56 °C, 60 s at 72 °C, with a final elongation of 10 min at 72 °C) was carried out in a 12·5-μL volume containing 1·25 μL of Tris–HCl, 1·25 μL of $MgCl_2$ (final concentration 2·5 mM), 1 μL of dNTP mix (final concentration 0·2 mM each), 0·25 μL of each primer (adaptors + three selective bases) at 10 μL, and 1 U of Ampli*Taq* Gold DNA polymerase (Applied Biosystems). Two primer combinations were chosen which resulted in clear bands of sufficient variability (EAGA–MGAC and EAGC–MCTG). For both combinations, the E– primer was labelled. PCR products were purified using columns of half to half Sephadex G50 at 5 % and Sephacryl S200. Finally 1·5 μL of the purified products was mixed with 10 μL of HiDi formamide and 0·1 μL Genescan ROX 500 size standards (Applied Biosystems), and electrophoresed on an ABI PRISM 3130 XL capillary sequencer (Applied Biosystems). PCR products from each primer pair *Eco*–*Mse* were run separately. Raw data were collected and sized using GENEMAPPER 3·7 (Applied Biosystems). Thirty-five samples were duplicated from the extraction step to check the reliability of experiments.

The AFLP profiles were scored using a semi-automated procedure to code presence (1) or absence (0) of bands. Bands were defined manually with GENEMAPPER for presence or absence of each band in each individual. The quality of bands was then automatically checked with an R script described in Herrmann *et al.* (2010), similar to that of Whitlock *et al.* (2008). This script removes bands with reproducibility lower than 2 % and screens AFLP phenotype identification (presence/absence of bands). AFLP fragments shorter than 50 bp and completely monomorphic fragments were discarded. With two pairs of primers, we could score 176 repeatable bands, of which 119 were polymorphic, and the total typing error was 1 %. This dataset was used to produce a dendrogram of genetic distances among individual stems in McIntire and Fajardo (2011).

Data analysis

The ability of a dataset to identify multilocus lineages and to estimate relatedness levels depends strongly on the diversity it harbours. We computed allele frequencies assuming Hardy–Weinberg equilibrium, and unbiased heterozygosity for each locus and analysed clonality using the GenAlEx version 6·41 (Peakall and Smouse, 2006) software.

Clonality. As AFLPs are dominant markers, only phenotypes [presence (1) or absence (0) of the band, called 'AFLP phenotypes' in the following] can be identified at each locus. We thus analysed the AFLP matrix looking for multilocus lineages, accounting for an error rate of 1 % (i.e. samples that differed by one AFLP band were assigned to the same multilocus lineage). This gave us eight multilocus lineages represented by two, three or four copies ('MLL' in Table 1). A new AFLP matrix was constructed by keeping only one replicate of each MLL, and this matrix was used for all further analyses.

To test the hypothesis that the MLLs we identified were not due to a low discrimination power of the dataset, we computed the probability of sampling two different genets with the same multilocus AFLP phenotype (probability of identity) for each of the MLL under the two extreme mating systems, i.e. random mating or complete selfing. We derived the probability of identity as the product of the probabilities of an identical AFLP phenotype at each locus, assuming loci are unlinked:

$$P(mlp_i) = \Pi[P(p_{il})]$$

with $P(mlp_i)$ being the probability of sampling two different genets with multilocus AFLP phenotype i and $P(p_{il})$ the probability of AFLP phenotype i at locus l. p_{il} is either $p(1)$ or $p(0)$ depending on the AFLP phenotype of multilocus AFLP phenotype i at locus l.

Under random mating, the probability of AFLP phenotype 0 (respectively, 1) at one locus is the frequency of AFLP phenotype 0, $f(0)$ [respectively, 1, $f(1)$] at that locus in the population $p(0) = f(0)$ [respectively, $p(1) = f(1)$]. Under complete selfing, $p(0) = q^2 + pq/2$ and $p(1) = p^2 + 3pq/2$, with p and q the frequency of allele 1 and 0 in the population as computed assuming Hardy–Weinberg equilibrium, i.e. $q = \sqrt{[f(0)]}$ and $p = 1 - q$. For this analysis, independence of markers was assessed by calculating a basic similarity index between all pairs of markers on the overall data (Gaudeul *et al.*, 2000). We discarded 16 markers that were >95 % or <5 % similar to another one.

Sibship structure. Sibship structure was analysed using the software COLONY (Wang, 2004; Wang and Santure, 2009; Jones and Wang, 2010). For each pair of samples, COLONY computes the likelihood of assignment into half sibs (one parent in common) or full sibs (same mother and father; note that in selfing organisms the mother and the father can be the same

TABLE 1. *Multilocus lineages (MLL) and sibship structure (derived from Fig. 2) of the clusters*

Cluster no.	*n*	MLL	FS		HS		NR
			$1 \geq P > 0{\cdot}9$	$0{\cdot}9 \geq P > 0{\cdot}75$	$1 \geq P > 0{\cdot}9$	$0{\cdot}9 \geq P > 0{\cdot}75$	
1	3	a + c				(ac) + b	
2	4	a + b + c			(abc) + d		
3	3	a + b	(ab) + c				
4	5	a + b + c	(abc) + d + e				
5	3		a + b				(ab) + c
16	5		a + c	(ac) + d	(ac) + b + e	b + d	
17	4	a + b	(ab) + d		(abd) + c		
18	6	b + d + e + f	(bdef) + a + c				
19	4		a + b		(ab) + c		(abc) + d
20	4		b + c				(bc) + a + d
21	5		a + b + c + e		(abce) + d		
22	6		a + b + c + d				(abcd) + e + f
23	4	a + b c + d	(ab) + (cd)				
24	4		a + b + d		(abd) + c		
25	6		a + b + c + d + e		(abcde) + f		

The different stems of a cluster were labelled 'a' to 'f'; e.g. in cluster no. 17, stems 'a' and 'b' belong to the same MLL and are full sibs of stem 'd'; the fourth stem (stem 'c') is half sib of the three other stems.

n, number of stems; MLL, stems belonging to the same multilocus lineage; FS, full sibs; HS, half sibs; NR, unrelated; *P*, average probability of two or more stems being half sib or full sib over the different runs.

individual). The maximum likelihood assignment was identified using the Markov Chain Monte Carlo method with the simulated annealing technique which allows typing errors to be taken into account. We set the parameters for a monoecious species with inbreeding and with female and male polygamy, and used a 0·005 per locus typing error. We chose the full-likelihood analysis method with 'medium precision', update of (formerly unknown) allele frequencies and no sibship size prior. We had no excluded paternity, maternity or sibship. After a few tests checking for convergence, we concluded that medium run length was enough to always attain convergence. Similarly to what Torimaru *et al.* (2007) obtained, different runs using the same parameters produced very similar but not identical results. We therefore computed the probability of each pair being half sib or full sib as the average of its likelihood of assignment into, respectively, half sibs and full sibs (separately) over ten independent runs (with different seed values for each run) with the dataset without duplicated MLL (69 samples). Pairs were identified as full sib (FS) or half sib (HS) only if the corresponding average probability was greater than 0·75; they were otherwise classified as unrelated (NR). All FS pairs had an average probability greater than 0·9. HS pairs with average probability between 0·9 and 0·75 were kept because some runs gave obviously aberrant results. Discarding these pairs does not change our main results.

Relatedness. We computed the pairwise relationship coefficient (r_{ij}; identity in state) following Hardy (2003) and using the software SPAGeDi 1·3 (Hardy and Vekemans, 2002). Although, with dominant markers, it is not possible to assess the exact relatedness between two given individuals, this estimator provides accurate estimates for groups of individuals relative to a reference population. For this analysis, we used the dataset without duplicated MLL and set the inbreeding coefficient (IC) at 0 (very different values of IC such as 0·1 and 0·5 gave almost identical results). Each pair was identified as 'within cluster' when both samples came from the same cluster, 'among clusters' when both samples came from different clusters, 'among single stems' when both samples came from single-stem trees and 'between clusters and single stems' in all other cases. The relatedness coefficient computed by SPAGeDi is relative to a 'reference population' (here the population under consideration). Negative values may thus be obtained, meaning that the two individuals are less related on average than random individuals from the population (Hardy, 2003).

The average relationship coefficient was then computed for full sibs (FS), half sibs (HS) and unrelated stems (NR). Following Hardy (2003), the value for NR corresponds to the reference population. We thus used the correction provided by eqn 6 of Hardy (2003) to compare the estimates to the theoretical values for FS (0·5) and HS (0·25). Pairwise relationship coefficients are not independent when individuals are related to each other within clusters. Full sibs were found essentially within 12 clusters, with the exception of one tree related to a cluster, which was included in the cluster for this analysis. We estimated the distribution of the average relationship coefficient for FS by sampling randomly one pair per cluster (i.e. with 12 independent values) and repeating the procedure 999 times. This gave us the distribution of r_{ij} for FS. The theoretical value (0·5) was then compared with this distribution and its probability was estimated. The same procedure was repeated for half sibs, for which seven groups of independent trees could be identified, and using a theoretical value of 0·25.

RESULTS

Genetic diversity of the population

The two selective primers allowed us to obtain 119 polymorphic bands with allele frequencies ranging from 0·015 to 0·759. The mean frequency of the dominant AFLP phenotype (D; presence of the band) is 0·23 but the distribution is skewed towards low values of D with 46 % of loci that have a $D \leq 0{\cdot}1$. The unbiased H_e is 0·179 (s.e. = 0·013; range 0·03–0·50), showing moderate genetic diversity in the sample.

Clonality

Identical copies were identified for eight MLL distributed in half of the clusters (seven out of 15; Table 1). No identical copies were identified between different clusters or trees, and in all clusters there were at least two different genets. The probability of obtaining identical AFLP phenotypes from different genotypes ranged from $4{\cdot}6 \times 10^{-12}$ to $9{\cdot}5 \times 10^{-18}$ for random mating and from $8{\cdot}6 \times 10^{-12}$ to $2{\cdot}0 \times 10^{-17}$ for selfing. These values indicate that the duplicated AFLP phenotypes come from vegetative multiplication and are not different individuals that could not be differentiated with our dataset.

Sibship structure

Of the 2346 possible pairs of individuals, 47 were identified as FS and 64 as HS (Fig. 2). The rest are unrelated pairs. Full sibs are almost exclusively found within clusters with the exception of one single tree (no. 14) grouping with part of cluster no. 25. Within a cluster, pairs that were not identified as FS are often HS. At least one pair of full sibs was identified in each cluster and unrelated stems are found in only five clusters, representing 9 % of the stems (Table 1). Between different clusters or trees, HS are not uncommon (43 pairs out of 2262) while FS are never found with the exception of tree no. 14 (five pairs out of 2262).

Relatedness

Relatedness between pairs of individuals ranged from −0·375 to 1·24. The means of all four categories were significantly different from each other ($r_{ij} = -0{\cdot}041$, s.e. = 0·003; −0·010, s.e. 0·006; 0·041, s.e. 0·013; and 0·657, s.e. 0·040 for 'among clusters', 'between clusters and single stems', 'among single stems' and 'within clusters', respectively). The average pairwise relationship coefficient within clusters was an order of magnitude higher than in the other three categories. Mostly unrelated individuals were found among clusters or among single stems. The distributions of 'within clusters' and 'between clusters and single stems' show two peaks (Fig. 3) with a peak centred on 0 and another mode centred on 0·8–0·9 showing that both highly related and unrelated individuals exist 'within clusters' and 'between clusters and single stems'. However, 'within

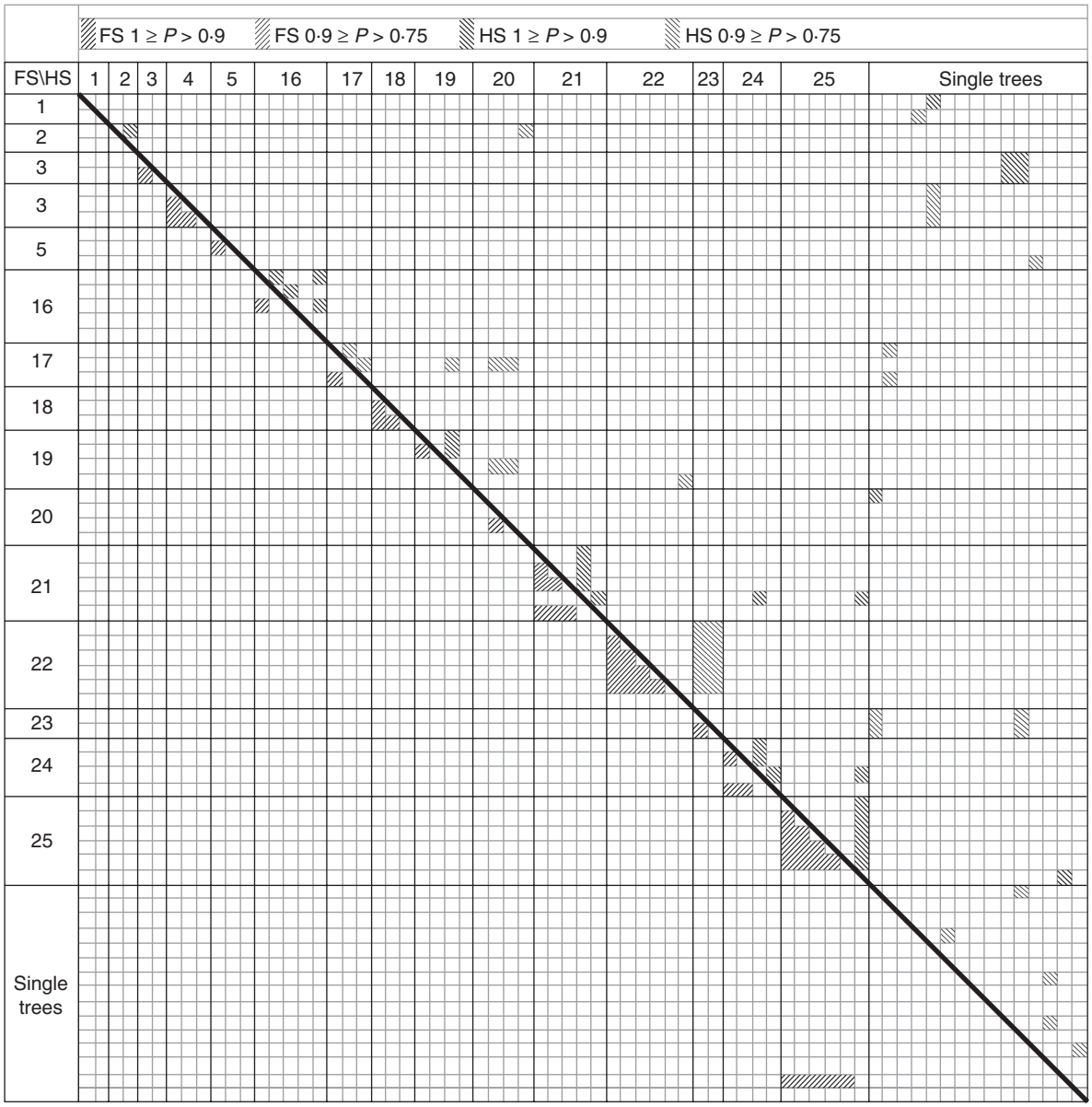

FIG 2. Sibship structure. Each cell represents the sibship assignment for each pair of samples [hatching: the two samples are full sibs or half sibs]. The different clusters are separated by vertical bars and labelled; the single trees are not individually labelled. Below diagonal, full sibs (FS); above diagona, half sibs (HS). Note, *P* stands for the average probability of two samples being half sib or full sib over the different runs (colour code: dark, $1 \geq P > 0{\cdot}9$; light, $0{\cdot}9 \geq P > 0{\cdot}75$).

clusters' the largest peak is 0·8 and 'between clusters and single stems', the dominant peak is 0. The corrected average pairwise relationship coefficients for FS and HS are significantly greater than the theoretical values of 0·5 ($P = 0{\cdot}001$) and 0·25 ($P = 0{\cdot}024$), respectively.

DISCUSSION

Although we identified some vegetative multiplication (MLLs) within the clusters, this was not the rule. MLLs were found in only seven of the 15 clusters and all clusters were composed of at least two different AFLP phenotypes. These clusters are not 'multi-trunk trees' (multiple trunks of a single genet) but tree clusters (as defined in Linhart and Tomback, 1985; Carsey and Tomback, 1994; Fajardo and McIntire, 2010). If we consider single trees or only one sample per cluster, the edge of second-growth forest is mostly composed of unrelated individuals with some half sibs, and very few full sib pairs. This suggests a large population of reproductive individuals and random distribution of seeds in the landscape. McIntire and Fajardo (2011), working with the same dataset, stated that there is little evidence of vegetative reproduction. The discrepancy comes from the fact that they did not take into account the error rate of the AFLP (1 %) such that samples differing by one locus were not identified as belonging to the same MLL. The AFLP technique is known to be error-prone (Bonin *et al.*, 2004; Herrmann *et al.*, 2010), thus it is important to take the error rate into account in the analyses.

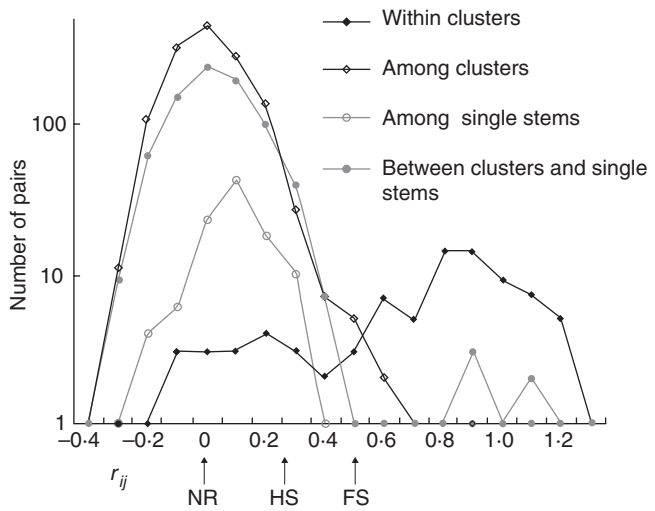

FIG 3. Distribution of pairwise relationship coefficients (r_{ij}). Each pair was identified as 'Within cluster' when both samples came from the same cluster, 'Among clusters' when both samples came from different clusters, 'Among single stems' when both samples came from single-stem trees and 'Between clusters and single stems' in all other cases (as indicated in the keys). Note that a log-scaled *y*-axis is used for greater clarity. The theoretical values for unrelated individuals (NR), half sibs (HS) and full sibs (FS) are indicated on the *x*-axis.

Within clusters, the pattern is very different. Trees (genets) are on average more related within than among clusters (by an order of magnitude). All clusters had at least one pair of half sibs, and most individuals within a cluster were full sibs or half sibs, very rarely unrelated (7·5 % of the stems in the clusters were unrelated to other stems). This confirms our hypothesis that genetic relatedness is high within clusters.

The probability of obtaining identical AFLP phenotypes from different genotypes was very low and the average unbiased heterozygosity is moderate (0·179), indicating that our dataset allows us to reliably address our main question. The average unbiased heterozygosity (computed supposing that the population is in Hardy–Weinberg equilibrium) is greater than the values obtained with isozymes on the same species [0·033–0·060, Premoli (2003); 0·0544–0·080, Mathiasen and Premoli (2010)]. However, isozymes are usually less polymorphic than AFLPs (Arnaud-Haond *et al.*, 2007) because monomorphic bands are discarded in the AFLP analysis. Our values fall within the medium range of diversity obtained with AFLPs ($H_e = 0{\cdot}198$ for *Eryngium alpinum*, Gaudeul *et al.*, 2000; $H_e = 0{\cdot}240$ for *Fagus sylvatica*, Jump *et al.*, 2006; $H_e = 0{\cdot}322$ on average for *Cryptomeria japonica*, Tsumura *et al.*, 2007). This is not surprising given that the population size is very large (wide ranging forest) and that in many tree species most of the genetic diversity lies within populations (Hamrick and Godt, 1989; Petit and Hampe, 2006).

High relatedness of individuals at very close geographic distances: possible explanations

Seeds of *N. pumilio* are dispersed by gravity or wind and most seeds fall beneath the tree crown (Rusch, 1993; Hoffmann, 1994; Heinemann *et al.*, 2000). Fajardo and McIntire (2010) suggest that seeds in this particular population were transported by gravity or run-off water. The distance between the edge of the old-growth forest and the second-growth edge can reach about 500 m (Fajardo and McIntire, 2010). Over such a distance, transport from a single source by wind, gravity or run-off water will result in a certain degree of dispersion, i.e. the seeds will be dispersed over an area certainly greater than the area covered by one cluster and therefore should lead to some mixing between seeds coming from different trees (Furnier *et al.*, 1987; Schuster and Mitton, 1991). Alternatively, the genetic structure found could be due to remnant trees belonging to the former mature forest that survived the fire. These trees have not been identified, but their existence cannot be ruled out at the time of recolonization.

Another explanation is animal dispersal and storage in caches (dyszoochory), similarly to what is found in some pine species (see Introduction). Although the Austral parakeet, *Enicognathus ferrugineus*, has been described as a seed eater of *N. pumilio*'s seeds (Díaz and Kitzberger, 2006), caching and endozoochory have not been observed (T. Kitzberger, Universidad Nacional del Comahue, Argentina, pers. comm.). Moreover, in pines, clusters occur throughout the populations whereas in *N. pumilio* multi-stemmed trees are only known from the edge of these second-growth, post-fire regeneration forests, not from natural, mature forests (Fajardo and McIntire, 2010). The animal disperser should be independent of the disturbance, fire, and multi-stemmed trees should be common in many different circumstances, which is clearly not the case.

McIntire and Fajardo (2011) showed that a two-step selection process was acting at the forest edge. First, at the establishment stage, facilitation favours seedlings growing in clusters, presumably because they create a 'wind-barrier' effect that decreases the negative consequences of desiccation from strong, spring Patagonian winds. Second, these seedlings/saplings clusters merge with time and become adult cluster trees which, on average, survive and grow better than single-stemmed trees. We have shown that the clusters are composed of at least one pair of highly related trees (full sibs) and more frequently of several trees with different levels of relatedness (full sibs + half sibs). If, as is the case in the ascidia *Botryllus schlosseri* (Grosberg and Quinn, 1986) and to a lesser degree in the pine species of the sugenus *Strobus* (see Introduction), merging is possible only if some stems in the clump are highly related, this would result in the pattern we observed. The occurrence of unrelated trees in some of the clusters might be due to a 'facilitation' effect, i.e. merging starts between kin trees and finally involves all trees (kin and non-kin) of the cluster (similarly to the 'rescue pollination' effect in pollen-tube growth where the germination of compatible pollen grains on a stigma allows non-compatible pollen tubes to develop; Adiwilaga and Brown, 1991). If merging requires at least one pair of related individuals, clusters of seedlings with related individuals would become tree clusters and clusters with only unrelated seedlings would result in single-stemmed trees.

High relatedness within multi-stemmed tree clusters

Whatever the origin of the clusters, our results show very high relatedness values. In *P. flexilis*, the average relatedness within clusters ranges between 0·19 (Schuster and Mitton,

1991) and 0·43 (Carsey and Tomback, 1994). Both values were obtained using isozyme markers. We obtained much higher values of average within-cluster relatedness using AFLP markers ($r_{ij} = 0{\cdot}66$). However, relatedness estimates were significantly higher than expected from assignments to full-sib and half-sib categories using the program COLONY (Wang, 2004) indicating that these two methods are not fully consistent. Relatedness estimates are affected by the inheritance mode of the markers, dominant markers leading to an upward bias of the r_{ij} estimator (Hardy, 2003). They are also affected by selfing and inbreeding. Selfing rates of 13 % have been reported in the three evergreen species of *Nothofagus* (*N. dombeyi*, *N. nitida* and *N. betuloides*; Premoli, 1997), whereas *N. pumilio* is deciduous. Although Mathiasen and Premoli (2010) describe *N. pumilio* as self-incompatible, no reproductive studies have been performed on this species and studies on other *Nothofagus* species (*N. dombeyi*, *N. nitida* and *N. obliqua*; the latter being deciduous) found that self-incompatibility was not complete, with up to 8·4 % of fruits forming seeds after autogamous self-pollination in *N. obliqua* (Riveros *et al.*, 1995). Moreover, significant heterozygote deficit was observed in some populations of *N. pumilio* using isozymes (Premoli, 2003; Mathiasen and Premoli, 2010) indicating partial selfing or inbreeding. *Northofagus pumilio* being monoecious, selfing is indeed possible and could have occurred on the forest edge just after the fire as a consequence of post-fire disturbance, and is especially plausible in solitary trees that survived the fire. As some trees originated from outcrossing (half sibs were found in eight of the 15 clusters; i.e. not all trees originated from selfing) and as we suspect selection for strong relatedness, only a small selfing rate is necessary to explain our results. However, selfing alone does not explain the high-relatedness coefficients. The theoretical value of the relationship coefficient between full sibs originating from selfing is also 0·5 and higher values are expected only if the parental generation is inbred.

In conclusion, merged tree clusters of *Nothofagus pumilio* at the second-growth forest edge contain multiple genets as well as multiple stems of the same genet. Merged genets are often strongly related (half sibs, full sibs or even higher). Possible explanations are selfing, inbreeding, abiotic and/or biotic dispersal conditions through which related seeds end up in the same place after dispersion, abiotic selection on clusters of seedlings at the edge of the second-growth forest, and kin selection requiring the merging of related individuals for better resource acquisition or better structural stability against wind.

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LITERATURE CITED

Adiwilaga KD, Brown CR. 1991. Use of 2N pollen-producing triploid hybrids to introduce tetraploid Mexican wild-species germ plasm to cultivated tetraploid potato gene pool. *Theoretical and Applied Genetics* **81**: 645–652.

Arnaud-Haond S, Duarte CM, Alberto F, Serrão EA. 2007. Standardizing methods to address clonality in population studies. *Molecular Ecology* **16**: 5115–5139.

Barrera MD, Frangi JL, Richter LL, Perdomo MH, Pinedo LB. 2000. Structural and functional changes in *Nothofagus pumilio* forests along an altitudinal gradient in Tierra del Fuego, Argentina. *Journal of Vegetation Science* **11**: 179–188.

Biernaskie JM. 2011. Evidence for competition and cooperation among climbing plants. *Proceedings of the Royal Society: B. Biological Sciences* **278**: 1989–1996.

Bond WJ, Midgley JJ. 2001. Ecology of sprouting in woody plants: the persistence niche. *Trends in Ecology and Evolution* **16**: 45–51.

Bonin A, Bellemain E, Bronken Eidesen P, Pompanon F, Brochmann C, Taberlet P. 2004. How to track and assess genotyping errors in population genetics studies. *Molecular Ecology* **13**: 3261–3273.

Carsey KS, Tomback DF. 1994. Growth form distribution and genetic relationships in tree clusters of *Pinus flexilis*, a bird-dispersed pine. *Oecologia* **98**: 402–411.

Cavieres LA, Fajardo A. 2005. Browsing by guanaco (*Lama guanicoe*) on *Nothofagus pumilio* forest gaps in Tierra del Fuego, Chile. *Forest Ecology and Management* **204**: 237–248.

Del Tredici P. 2001. Sprouting in temperate trees: a morphological and ecological review. *Botanical Review* **67**: 121–140.

Devi N, Hagedorn F, Moiseev P, *et al*. 2008. Expanding forest and changing growth forms of Siberian larch at the Polar Urals treeline during the 20th century. *Global Change Biology* **14**: 1581–1591.

Díaz S, Kitzberger T. 2006. High *Nothofagus* flower consumption and pollen emptying in the southern South America austral parakeet (*Enicognathus ferrugineus*). *Austral Ecology* **31**: 759–766.

Escaravage N, Questiau S, Pornon A, Doche B, Taberlet P. 1998. Clonal diversity in a *Rhododendron ferrugineum* L. (Ericaceae) population inferred from AFLP markers. *Molecular Ecology* **7**: 975–982.

Fajardo A, González ME. 2009. Replacement patterns and species coexistence in an Andean *Araucaria – Nothofagus* forest. *Journal of Vegetation Science* **20**: 1176–1190.

Fajardo A, de Graaf R. 2004. Tree dynamics in canopy gaps in old-growth forests of *Nothofagus pumilio* in Southern Chile. *Plant Ecology* **173**: 95–106.

Fajardo A, McIntire EJB. 2010. Merged trees in second-growth, fire origin forests in Patagonia, Chile: positive spatial association patterns and their ecological implications. *American Journal of Botany* **97**: 1424–1430.

Fajardo A, McIntire EJB. 2011. Under strong niche overlap conspecifics do not compete but help each other to survive: facilitation at the intraspecific level. *Journal of Ecology* **99**: 642–650.

Furnier GR, Knowles P, Clyde MA, Dancik BP. 1987. Effects of avian seed dispersal on the genetic structure of whitebark pine populations. *Evolution* **41**: 607–612.

Galeuchet DJ, Holderegger R, Rutishauser R, Schneller JJ. 2002. Isozyme diversity and reproduction of *Typha minima* populations on the upper River Rhine. *Aquatic Botany* **74**: 19–32.

Gaudeul M, Till-Bottraud I, Taberlet P. 2000. Genetic diversity in an endangered alpine plant, *Eryngium alpinum* L. (Apiaceae), inferred from AFLP markers. *Molecular Ecology* **9**: 1625–1637.

Grosberg RK, Quinn JF. 1986. The genetic control and consequences of kin recognition by the larvae of a colonial marine invertebrate. *Nature* **322**: 456–459.

Hamrick JL, Godt MJ. 1989. Allozyme diversity in plant species. In: Brown A, Kahler CM, Weir BS, eds. *Plant population genetics breeding and genetic resources.* Sunderland, MA: Sinauer.

Hamrick JL, Nason JD. 1996. Consequences of dispersal in plants. In: Rhodes OE, Chesser RK, Smith MH, eds. *Population dynamics in ecological space and time.* Chicago, IL: University of Chicago Press.

Hardy OJ. 2003. Estimation of pairwise relatedness between individuals and characterization of isolation-by-distance processes using dominant genetic markers. *Molecular Ecology* **12**: 1577–1588.

Hardy OJ, Vekemans X. 2002. SPAGEDi: a versatile computer program to analyse spatial genetic structure at the individual or population levels. *Molecular Ecology Notes* **2**: 618–620.

Harper JL. 1977. *Population ecology of plants*. London: Academic Press.

Heinemann K, Kitzberger T, Veblen TT. 2000. Influences of gap microheterogeneity on the regeneration of *Nothofagus pumilio* in a xeric old-growth forest of north-western Patagonia, Argentina. *Canadian Journal of Forest Research* **30**: 25–31.

Herrmann D, Poncet BN, Manel S, *et al*. 2010. Selection criteria for scoring amplified fragment length polymorphisms (AFLPs) positively affect the reliability of population genetic parameter estimates. *Genome* **53**: 302–310.

Hoffmann AE. 1994. *Flora silvestre de Chile. Zona araucana. Arboles, arbustos y enredaderas leñosas*. Santiago, Chile: Ediciones Claudio Gay.

Jones AG, Small CM, Paczolt KA, Ratterman NL. 2010. A practical guide to methods of parentage analysis. *Molecular Ecology Resources* **10**: 6–30.

Jones OR, Wang JL. 2010. COLONY: a program for parentage and sibship inference from multilocus genotype data. *Molecular Ecology Resources* **10**: 551–555.

Jorgensen SM, Hamrick JL. 1997. Biogeography and population genetics of whitebark pine, *Pinus albicaulis*. *Canadian Journal of Forest Research* **27**:1574–1585.

Jump AS, Hunt JM, Martínez-Izquierdo JA, Peñuelas J. 2006. Natural selection and climate change: temperature-linked spatial and temporal trends in gene frequency in *Fagus sylvatica*. *Molecular Ecology* **15**: 3469–3480.

Linhart YB, Tomback DF. 1985. Seed dispersal by nutcrackers causes multi-trunk growth form in pines. *Oecologia* **67**: 107–110.

McIntire EJB, Fajardo A. 2011. Facilitation within species: a possible origin of group selected superorganisms. *The American Naturalist* **178**: 88–97.

Mascareño A. 1987. Evaluación de ensayos de semillación y regeneración de lenga (*Nothofagus pumilio* (Poepp. et Endl. Krasser)) bajo diferentes tratamientos a la cama de semillas en la Reserva Forestal Trapananda, Coyhaique, XI Región. *Facultad de Ciencias Forestales*, Universidad Austral de Chile, Valdivia, Chile.

Mathiasen P, Premoli AC. 2010. Out in the cold: genetic variation of *Nothofagus pumilio* (Nothofagaceae) provides evidence for latitudinally distinct evolutionary histories in austral South America. *Molecular Ecology* **19**: 371–385.

Peakall R, Smouse PE. 2006. GENALEX 6: genetic analysis in Excel: population genetic software for teaching and research. *Molecular Ecology Notes* **6**: 288–295.

Petit RJ, Hampe A. 2006. Some evolutionary consequences of being a tree. *Annual Review of Ecology, Evolution, and Systematics* **37**: 187–214.

Premoli AC. 1997. Genetic variation in a geographically restricted and two widespread species of South American *Nothofagus*. *Journal of Biogeography* **24**: 883–894.

Premoli AC. 2003. Isozyme polymorphisms provide evidence of clinal variation with elevation in *Nothofagus pumilio*. *Journal of Heredity* **94**: 218–226.

Riveros M, Parades MA, Rosas MT, *et al*. 1995. Reproductive biology in species of the genus *Nothofagus*. *Environmental and Experimental Botany* **35**: 519–524.

Rusch V. 1993. Altitudinal variation in the phenology of *Nothofagus pumilio* in Argentina. *Revista Chilena de Historia Natural* **66**: 131–141.

Schuster WSF, Mitton JB. 1991. Relatedness within clusters of a bird-dispersed pine and the potential for kin interactions. *Heredity* **67**: 41–48.

Thomson JD, Herre EA, Hamrick JL, Stone JL. 1991. Genetic mosaics in strangler fig trees: implications for tropical conservation. *Science* **254**: 1214–1216.

Tomback DF, Holtmeier F-K, Mattes H, Carsey KS, Powell ML. 1993. Tree clusters and growth form distribution in *Pinus cembra*, a bird-dispersed pine. *Arctic, Antarctic and Alpine Research* **25**: 374–381.

Torimaru T, Tani N, Tsumura Y, Nishimura N, Tomaru N. 2007. Effects of kin-structured seed dispersal on the genetic structure of the clonal dioecious shrub *Ilex leucoclada*. *Evolution* **61**: 1289–1300.

Tsumura Y, Kado T, Takahashi T, Tani N, Ujino-Ihara T, Iwata H. 2007. Genome scan to detect genetic structure and adaptive genes of natural populations of *Cryptomeria japonica*. *Genetics* **176**: 2393–2403.

Veblen TT, Donoso C, Kitzberger T, Rebertus A. 1996. Ecology of Southern Chilean and Argentinean *Nothofagus* forests. In: Veblen TT, Hill RS, Read J, eds. *The ecology and biogeography of* Nothofagus *forests*. New Haven, CT: Yale University Press.

Vos P, Hogers R, Bleeker M, *et al*. 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* **23**: 4407–4414.

Wang J. 2004. Estimating pairwise relatedness from dominant genetic markers. *Molecular Ecology* **13**: 3169–3178.

Wang J, Santure AW. 2009. Parental and sibship inference from multilocus genotype data under polygamy. *Genetics* **181**: 1579–1594.

Wardle P. 2008. New Zealand forest to alpine transitions in global context. *Arctic, Antarctic and Alpine Research* **40**: 240–249.

West SA, Griffin AS, Gardner A. 2007. Social semantics: altruism, cooperation, mutualism, strong reciprocity and group selection. *Journal of Evolutionary Biology* **20**: 415–432.

Whitlock R, Hipperson H, Mannarelli M, Butlin RK, Burke T. 2008. An objective, rapid and reproducible method for scoring AFLP peak-height data that minimizes genotyping error. *Molecular Ecology Resources* **8**: 725–735.