Ciencia e Investigación AGRARIA

Cien. Inv. Agr. 36(2):229-238. 2009
www.rcia.puc.cl

RESEARCH PAPER

Proximal causes of genetic variation between and within populations of raulí (*Nothofagus nervosa*)

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Abstract

B. Carrasco. **M. Garnier. L. Eaton. R. Guevara, and M. Carú. 2009. Proximal causes of genetic variation between and within populations of raulí (*Nothofagus nervosa*). Cien. Inv. Agr. 36(2):229-238.** Random Amplified Polymorphic DNA (RAPD) markers were used to assess the genetic diversity of 587 individuals, belonging to 22 populations of *Nothofagus nervosa* that were distributed through the Coastal (38°S to 41°S) and Andes Mountains in Central-Southern Chile (36°S to 40°S). The objective of this study was to complement the genetic inferences previously determined by isozyme analysis, in order to obtain more accurate genetic diversity estimations. We scored 81.8% of the polymorphic loci of the samples tested. The average incidence of genetic polymorphism within populations was high, with values ranging between 33% and 63%. Analysis of molecular variance (AMOVA) showed most of the genetic variation was distributed within populations (87.6%), but F_{ST} values ($F_{ST} = 0.124$; $p < 0.00001$) indicated that there was also a significant difference among populations. A discriminant analysis revealed three geographically defined groups and showed that 14 loci explained 87.2% of the genetic differentiation among *N. nervosa* populations. Watterson's neutrality test and Ohta's two-locus analysis of linkage disequilibrium (LD) both suggested that stochastic demographic and environmental factors can partially explain the loci variation observed in the RAPDs. The role of the last glaciations, as well as some conservation and breeding strategies, may have influenced current genetic variation and fragmentation in this species.

Key words: Genetic structure, *Nothofagus,* RAPD markers.

Introduction

Nothofagus is an important genus of the temperate forests of South America. Traditionally, the genus *Nothofagus* was classified as a member of the Fagaceae family. However, this genus has recently been included in the monogeneric family Nothofagaceae, based on morphological, developmental, and biogeographical studies (Veblen *et al*., 1996; Souza *et al*., 2000). In Chile, there are nine species of *Nothofagus*, including *N. nervosa* (Phil.) Dim. et Mil. ("raulí"), which is a large deciduous tree that grows in the Chilean Andes from 36°S to just south of 40°S. *Nothofagus nervosa* is also found in the Coastal Mountains, from 38°S to 41°S, and has been identified in a few stands in Argentina near the Chilean border (Donoso. 1995). *Nothofagus nervosa* grows on the lower slopes of the Andes,

Received 15 September 2008. Accepted 27 December 2008.
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at altitudes from 100 to 1200 m, forming mixed forests with *N. dombeyi* (Mirb.) Oerst. ("coigüe") and sometimes *N. obliqua* (Mirb.) Oerst. ("roble"). However, "raulí" generally grows at higher altitudes than "roble".

Nothofagus nervosa is a monoecious species that is largely outcrossing, with predominantly wind-dispersed pollen and seeds (Donoso, 1995). Most of the "raulí" populations from the Coastal Mountain Range have become extinct, while the remaining populations have been reduced to dispersed and small patches, due to intensive use of the plant as a wood source. Genetic differentiation may be expected, considering the geographic separation and isolation of some natural populations of *N. nervosa* in both the Andes and coastal mountains. It is known that habitat fragmentation can result in a loss of genetic variability and, in the long term, can cause genetic differentiation between populations, resulting in a loss of fitness (Garcia *et al*., 2008). It would be surprising if the natural populations of "raulí" have escaped this process during their natural history.

Previous reports (Carrasco, 1998; Carrasco and Eaton, 2002) based on 10 isozyme loci have revealed a moderate level of variability between populations of "raulí". However, the data obtained from these studies cannot support any realistic inference about the evolutionary process and conservation plans for this species, because of the low level of genetic resolution given by isozyme loci.

In order to complement the genetic inferences previously determined by isozyme analysis (Carrasco and Eaton, 2002), the genetic diversity between and within populations of raulí was studied using an alternative molecular marker, such as randomly amplified polymorphic DNA (RAPD) (Welsh and McCLelland, 1990; Williams *et al*.,1990; Hadrys *et al*., 1992), and a larger sample size of 586 individuals from 22 distinct populations of *N. nervosa* from central and southern Chile (Figure 1). The populations studied were found in both the Andean and Coastal mountains, representing the complete natural distribution of the species in Chile. Therefore, the data obtained from this study will permit us to examine the possible causes of the observed pattern of genetic variation, and to compare our results with other studies that have used dominant molecular markers in forest species.

Materials and methods

Populations studied

The geographic locations of the 22 populations of *N. nervosa* sampled are indicated in Table 1 and Figure 1. A total of 586 individuals were analyzed in this study. Each individual was sampled randomly, with at least 30 m between trees, to minimize sampling among relatives. Terminal and lateral branch samples were collected, including at least six new leaves from each individual. Leaves were frozen in liquid nitrogen until they were processed for DNA extraction.

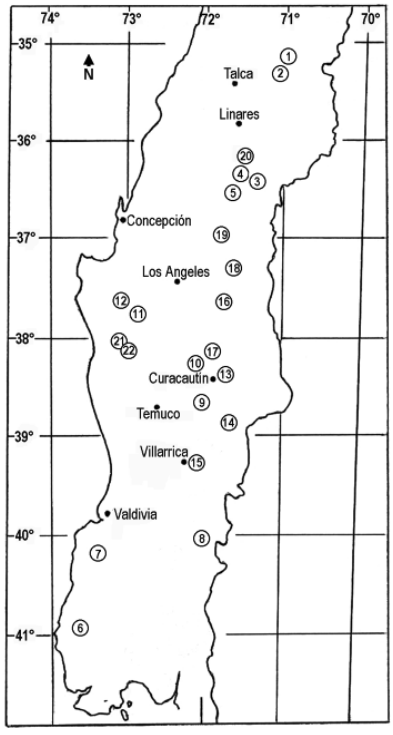

Figure 1. Map of central Chile, showing the actual range of *Nothofagus nervosa*. Numbers indicate the studied populations: 1 Aguas Frias, 2 Vilches, 3 San Fabian Alto, 4 San Fabian Bajo, 5 Recinto, 6 El Colegual, 7 Las Trancas, 8 Maihue, 9 Cherquenco, 10 Selva Oscura, 11 Angol, 12 Nahuelbuta, 13 Curacautin, 14 Melipeuco, 15 Villarica, 16 Ralco, 17 Laguna, Malleco, 18 Antuco, 19 Monte León, 20 Bullileo, 21, Villa Araucária, 22 Capitán Pastenes.

Table 1. Geographic data of populations of *Nothofagus nervosa* ("raulí") sampled to determine genetic variation by Random Amplified Polymorphic DNA.

Populations	Mountain Ranges	Latitude South	Longitude West	Altitude m
1. Aguas Frías	Andes	35°21'	71°04'	650
2. Vilches	Andes	35°27'	71°08	850
3. San Fabian Alto	Andes	36°39'	71°33'	750
4. San Fabian Bajo	Andes	36°34'	71°38'	450
5. Recinto	Andes	36°49'	71°41'	750
6. El Colegual	coastal	40°56'	73°28'	238
7. Las Trancas	coastal	40°15'	73°20'	150
8. Maihue	Andes	40°13'	71°59'	250
9. Cherquenco	Andes	38°40'	71°57'	300
10. Selva Oscura	Andes	38°19'	72°05'	350
11. Angol	coastal	37°47'	72°50'	530
12. Nahuelbuta	coastal	37°40'	73°13'	950
13. Curacautín	Andes	38°27'	71°44'	750
14. Melipeuco	Andes	38°51'	71°38'	750
15. Villarrica	Andes	39°27'	72°13'	310
16. Ralco	Andes	37°40'	71°40'	150
17. Laguna Malleco	Andes	38°14'	71°50	850
18. Antuco	Andes	37°23'	71°32'	710
19. Monte León	Andes	37°01'	71°49'	650
20. Bullileo	Andes	36°26'	71°37'	720
21. Villa Araucanía	coastal	38°05'	73°10'	250
22. Capitán Pastene	coastal	38°10'	72°58'	280

DNA isolation and PCR amplification

High quality DNA was obtained using a CTAB (hexadecyltrimethylammonium bromide) based protocol, adapted from Doyle (1991). PCR amplification was performed in a GeneAmp 2400 (Perkin Elmer Co., Norwalk, CT, USA) thermal cycler, with the following program: 5 min predenaturation at 94°C followed by 40 cycles of 1 min denaturation at 92°C, 1 min annealing at 36°C, and 1 min extension at 72°C, with a final extension of 5 min at 72°C. The amplification reaction contained 10 mM Tris, 50 mM KCl, 2 mM $MgCl_2$, 0.1 mM of each dNTP, 0.4 mM primer, 2.5 units of Taq polymerase (GIBCO-BRL) and 20 ng template DNA. A negative control without DNA template was added to each run to test for contamination. Ten arbitrary primers were screened (kit OPBF, Operon Technologies, Alameda. CA, USA), but only two primers (OPBF-02, 5'GAC ACA CTC C3' and OPBF-18, 5'AGC CAA GGA C3') produced repeatable and consistent band patterns and were therefore used for further analyses.

The amplification products were separated by electrophoresis on 1.4% agarose gels and visu-

alized under UV light after staining in ethidium bromide. PCR reactions and electrophoreses were repeated at least twice to assure the reproducibility of the bands.

Data scoring and analysis

The size of the PCR products was determined using the Kodak Digital Science ID program (Eastman Kodak Co., Rochester, NY, USA). Only reproducible bands were scored as present (1) or absent (0). Two assumptions were made in RAPD statistical analyses: (1) co-migrating bands were homologous; and (2) different band positions represented different loci. To estimate polymorphism parameters in the populations, the presence/absence data matrix was analyzed with POPGENE 1.31 (Yeh *et al*., 1999). This program calculates the polymorphic loci percentage (P). Shannon's index (S) is known as phenotypic diversity [S = - *pi* (log 2 *pi*)], where *pi* is the proportion of individuals having a band at a particular locus, averaged by the number of polymorphic loci in each population. A large score indicates large genetic diversity in the population. Shannon's index and 95% confidence intervals of Shannon's index were estimated using 1000 bootstrap samples. Nei's gene diversity (H) was also estimated (Nei, 1973, 1978).

The distribution of genetic diversity within and among populations (F_{st}) was calculated using the analysis of molecular variance (AMOVA, Excoffier *et al*., 1992). A discriminant analysis (DA) was performed in order to determine relationships among populations and to determine which loci explain those differences. For this analysis, Statistica 4.5 software (StatSoft Inc., Tulsa, OK USA) was used, and only polymorphic loci were included. The DA was applied because is a more sensitive method of revealing relationships among populations, as it does not impose a hierarchical structure, such as a dendrogram, and it allows misclassified individuals and populations to be identified.

The relationship between genetic distance and geographic distance was analyzed by a Mantel test (Mantel, 1967), using the Mantel Nonparametric Test Calculator package, version 2.00 (Liedloff, 1999).

To detect the possible effects of selection/genetic drift on inter-population allele distribution, the Evens-Watterson test for neutrality (1000 permutations per test) was applied using POPGENE version 1.31 (Yeh, *et al*., 1999). Linkage disequilibrium (LD) was estimated using Ohta's two-locus analysis for subdivided populations (Ohta, 1982), included in the GENEPOP package version 1.2 (Raymond and Rousset, 1995). A total of 528 loci comparisons ([N (N-1)$\cdot^{-2}$]) were performed, where N = 33 was the number of loci analyzed.

Ohta's approach makes two partitions of the total variance of dilocus LD (D^2_{IT}), within-population and between-population components: D^2_{IS} (average LD within subpopulations) and D^2_{ST} (variance of expected "chromosome" frequencies among subpopulations), and D'^2_{IS} (variance of the observed "chromosome" frequencies in subpopulations from the observed totals) and D'^2_{ST} (variance of the observed totals from the average expected frequencies of the total population) (Ohta, 1982). Ohta's model predicts that LD is due to random drift if $D^2_{ST} > D^2_{IS}$ and $D'^2_{IS} > D'^2_{ST}$, and that LD is due to epistatic selection if $D^2_{ST} < D^2_{IS}$ and $D'^2_{IS} < D'^2_{ST}$. The variances were calculated by adding the squared deviations and calculating weighted averages (Ohta, 1982).

Results

A total of 33 putative RAPD loci were identified from the 586 individuals analyzed from 22 natural populations. The number of scored bands and size fragments amplified with the different primers ranged from 15 bands to 18 bands, with sizes ranging from 375 to 1900 bp and 650 to 1700 bp, respectively.

As a dominant marker type, RAPDs are visualized by the presence or absence of a band. Therefore, it was assumed that absence of a band indicated that the individual was homozygous for the recessive allele. Calculation of genetic diversity values also assumes that the population is in Hardy-Weinberg equilibrium. At the species level, out of the 33 loci, 27 (81.8%) were polymorphic and 6 loci were consistently monomorphic. Shannon's Index (S) and gene diversity were S = 0.23 and H = 0.15, respectively. At the population level, all statistical tests had decreased average values. The average percentage of polymorphic loci per population and Shannon's Index ranged from P = 33.3% to 63.6% and S = 0.161 to 0.284, respectively (Table 2). Nahuelbuta showed the lowest values (P = 33.3%. S = 0.161), while Recinto displayed the highest (P = 63.5 S = 0.284).

Table 2. Shannon's index and polymorphic loci for 33 RAPD loci in 22 populations of *Nothofagus nervosa* ("raulí") from southern Chile.

Populations	Group	Shannon's index	Polymorphic loci (%)
1. Aguas Frias	A	0.24	45.5
2. Vilches	A	0.24	45.5
3. San Fabian Alto	A	0.26	57.6
4. San Fabian Bajo	A	0.24	54.6
5. Recinto	A	0.28	63.5
11. Angol	A	0.21	42.4
14. Melipeuco	A	0.21	45.5
16. Ralco	A	0.25	51.5
18. Antuco	A	0.20	48.5
19. Monte León	A	0.22	54.6
20. Bullileo	A	0.18	45.5
Average		0.24	51
6. El Colegual	B	0.23	51.5
8. Maihue	B	0.26	54.6
9. Cherquenco	B	0.25	48.5
10. Selva Oscura	B	0.24	57.6
12. Nahuelbuta	B	0.16	33.3
13. Curacautin	B	0.24	54.6
15. Villarica	B	0.21	45.5
17. Laguna Malleco	B	0.24	42.4
21. Villa Araucaria	B	0.21	42.4
22. Capitán Pastene	B	0.22	54.6
Average		0.23	48
7. Las Trancas	C	0.22	52

Genetic diversity between populations

The mean value of Nei's genetic distance was D = 0.047. In agreement with these results, AMOVA showed that most of the genetic variation was found within populations (87.6%), and moderate variation occurs between populations (F_{ST} = 0.124, p < 0.00001) (Table 3).

87.5 % of the variability (p < 0.05). The DA was able to distinguish three population clusters (Figure 2), which were identified as A, B and C. Cluster A included the populations located in the northern area of the natural distribution, through the Andean Mountains: Aguas Frías (1), Vilches (2), San Fabían Alto (3), San Fabian Bajo (4), Recinto (5), Angol (11), Melipeuco (14), Ralco

Table 3. Analysis of molecular variance (AMOVA), using pairwise difference for 22 populations of *Nothofagus nervosa* ("raulí") and 33 RAPD loci.

Source of variation	d.f.	Sum of squares	Variance components	Variation %	p
Among populations	21	102.578	0.14519	12.4	<0.000001
Within populations	565	578.764	1.02436	87.6	<0.000001

Fixation Index F_{ST}= 0.124. Significance tests over 1000 permutations.

The DA showed that 14 RAPD loci were sufficient for consistent clustering and 100% of reduction in the classification error (Figure 2). Moreover, a single discriminant function explained

(16), Antuco (18), Monte León (19), and Bullileo (20). The Angol (11) population was the only one located in the Coastal Mountains, and Melipeuco (14) was the southernmost population.

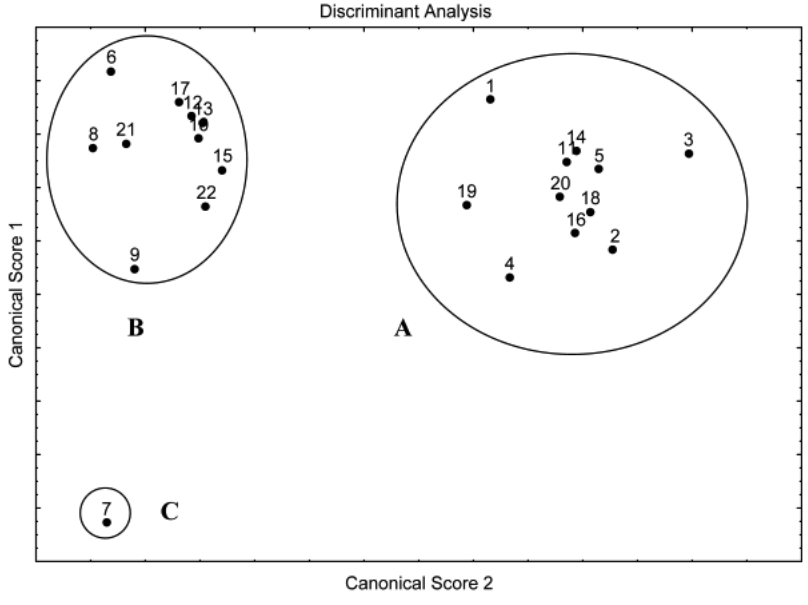

Figure 2. Discriminant analysis of 33 RAPD loci and 22 natural populations of *Nothofagus nervosa* ("raulí"). VC1 = canonical variable 1 and CV2 = canonical variable 2. The 22 populations were separated into 3 clusters: Cluster A included the populations located in the northern area through the Andean Mountains, Cluster B included the populations located in the southern area through the Andean Mountains, and Cluster C included only one population, Las Trancas (7), from the costal Mountains.

Cluster B included the southern populations located in the Andean Mountains: Maihue (8), Cherquenco (9), Selva Oscura (10), Curacautín (13), Villarrica (15) and Laguna Malleco (17). Cluster C also included populations from the Coastal Mountains: El Colegual (6), Nahuelbuta (12), Villa Araucanía (21), and Capitán Pastenes (22).

Las Trancas (7), a sample from the Coastal Mountains, was the only population assigned to cluster C.

In agreement with DA, the Mantel test for genetic and geographic distance indicated that raulí populations showed a consistent geographic pattern for genetic variability ($r = 0.469$. $p < 0.01$).

Linkage disequilibrium

The Evens-Watterson test was not significant for the any of the 33 putative RAPD loci analyzed in this study, indicating that they may be considered as selectively neutral (data not shown). Ohta's test for all 528 loci combinations gave the following average values for the components of total variance of dilocus LD: $D^2_{IS} = 0.0054$; $D^2_{ST} = 0.0738$; $D'^2_{IS} = 0.294$; $D'^2_{ST} = 0.0014$; $D^2_{IT} = 0.2954$. These values are consistent with the expected pattern under Ohta's model in which the relationships among loci are produced by limited migration and genetic drift, but not by epistatic natural selection ($D^2_{IS} < D^2_{ST}$ and $D'^2_{IS} > D^2_{ST}$). It should be noted that there is essentially no dilocus disequilibrium within populations ($D^2_{IS} = 0.0054$), and that almost all the variation in D_{IT} is due to differences in allele frequencies among populations ($D'^2_{IS} = 0.294$), not to systematic differences in "chromosome" frequencies ($D'^2_{ST} = 0.0014$).

Discussion

There has been increasing interest in the use of DNA-based molecular markers for a number of applications in population genetics, conservation and tree improvement. RAPD markers have been used in a wide variety of genetic studies of trees, including *Picea abies* (L.) Karst. (Bucci and Menozzi, 1995), *P. sitchensis* (Bong.) Carr. (Van Den Ven and McNicol, 1995), *Quercus petraea* (Matt.) Liebl. (Le Corre *et al*., 1997), *Andropogon gerardii* Vitman (Gustafson *et al*., 1999) and *Pinus leucodermis* Antoine (Bucci *et al*., 1997).

The genetic diversity of "raulí" natural populations ($H = 0.15$) was low, compared to other plant species. Nybom *et al*. (2004) showed higher genetic diversity values with dominant molecular markers in other plant species ($H_{RAPD} = 0.22$, $H_{AFLP} = 0.23$, $H_{ISSR} = 0.22$). Similarly, RAPD diversity was lower, compared to other long-lived forest species. For example, the mean value of Shannon's index ($S = 0.23$) was considerably lower than those reported for *Araucaria araucana* (Molina) K. Koch ($S = 0.65$, Bekessy *et al*., 2002), *Fitzroya cupressoides* (Molina) Johnston ($S = 0.54$, Allnutt *et al*., 1999) and *Populus tremuloides* Michx ($S = 0.65$, Yeh *et al*., 1995). The lower RAPD genetic diversity observed in "raulí" populations may be a reflection of the intense exploitation that these populations have suffered during the last century. Almost all natural populations of "raulí" have been replaced by exotic forest trees such as *Pinus radiata* D. Don and *Eucaliptus* sp.

Mattioni *et al*. (2002) found that 87% of RAPD loci were polymorphic, with a low identity ($I = 0.751$) among 61 individuals of "raulí". Our results were similar for the polymorphism data (81.8%), but very different for identity ($I = 0.96$). This discrepancy can be explained by our much larger sample size ($N = 586$ individuals). Interestingly, the "raulí" populations revealed less genetic diversity for RAPD markers ($H = 0.15$) than for allozyme analysis ($H = 0.28$; Carrasco and Eaton, 2002). There are several possible explanations for the difference between variability patterns identified through isozymes and RAPDs. There are intrinsic differences between these two types of genetic markers; RAPD loci have a maximum of two alleles, while isozymes frequently have more than two. Further, heterozygote genotypes can be identified with co-dominant isozymes, while they may only be estimated with dominant RAPDs. Second, the selection regime of RAPDs may be different from that of allozymes (Begun and

Aquadro, 1993; Baruffi *et al*., 1995). Previous studies have highlighted the non-neutrality of allozymes (Karl and Avise, 1992), specifically that allozymes are expected to be more susceptible to natural selection than RAPDs. Third, some previous studies have revealed that genetic diversity is greater at RAPD loci than at allozyme loci, suggesting that mutation rates are higher at the RAPD loci (Peakall *et al*., 1995). Finally, the coverage of the genome is expected to be different between allozymes and RAPDs. In the present study, we examined 33 random putative loci, while only 10 loci were examined in the allozyme study reported by Carrasco and Eaton (2002).

A significant genetic structure was detected for the genetic diversity of RAPDs. AMOVA showed a moderate differentiation between populations (F_{ST} = 0.12); this value was lower than the average found in plants for dominant molecular markers (RAPDs F_{ST} = 0.34; AFLP F_{ST} = 0.35; ISSR F_{ST} = 0.35; Nybom, 2004). However, the genetic differentiation among "raulí" populations was similar than other outbreed and wind pollinated Chilean forest species, such as *A. araucana* (RAPDs F_{ST}=0.128; Bekessy *et al*., 2002) and *Pilgerodendron uviferum* (D. Don) Florin (RAPDs F_{ST} = 0.186; Allnutt *et al*., 2003), but smaller than that of *Gomortega keule* (ISSR Fst = 0.27; García *et al*., 2008).

Moreover, there was an overall correlation between geographic and genetic distance, and the discriminant analysis showed that the populations grouped into three separate geographic zones (Figure 2). Cluster A included forests in the northern part of the species' range in the Andes and the northern part of the Nahuelbuta (coastal) Mountains. Cluster B included populations from more southern parts of both mountain ranges, while cluster C was composed of the population from Las Trancas, near the southern limit of the species' current range. Clusters A and B included populations from the Nahuelbuta Mountains (populations 11, 12, 21 and 22), which coincides with palynological evidence suggesting that this mountain range was a refuge for this species during the last glaciation (Villagran 1991, 2001). Cluster C probably represents a second refuge during the last glaciation, in agreement with allozyme variability (Carrasco and Eaton, 2002). Surprisingly, the southernmost population, El Colegual (6), grouped with the populations from the southern Andes (cluster B), and did not appear as an isolated entity, as was the case with allozymes.

Based upon the groupings of populations using RAPDs and isozymes, we suggest that conservation and breeding programs for this species should include populations from at least the three clusters described in this study.

In order to examine the evolutionary causes of the observed genetic pattern, we applied a neutrality test and analyzed linkage disequilibrium according to Ohta's approach. We found evidence that migration and genetic drift are the most important evolutionary forces involved in the genetic structure of natural populations of "raulí". First, the neutrality test showed no evidence for selective forces on the RAPD loci, implying that these loci would have evolved only under genetic drift. Second, the D-statistics developed by Ohta (1982) to analyze causes of non-random associations of alleles did not show any evidence of systematic selection. Specifically, no allele combination was favored in particular geographic areas. This suggests that the observed non-systematic linkage disequilibrium is likely due to limited gene flow and genetic drift, without a notable contribution of selection. Our results are a strong argument in favor of the hypothesis that stochastic demographic and random environmental factors have been the proximal causes of variation in RAPD loci in natural populations of "raulí".

Acknowledgements

We thank Mr. Oscar Chandía for assistance in the field. This work was funded by Fondecyt project N° 1990933.

Resumen

B. Carrasco, M. Garnier, L. Eaton, R. Guevara y M. Carú. 2009. Causas proximales de la variación genética entre y dentro de poblaciones de raulí (*Nothofagus nervosa*). Cien. Inv. Agr. 36(2):229-238. Se evaluó la diversidad genética de 587 individuos pertenecientes a 22 poblaciones naturales de raulí (*Nothofagus nervosa*), distribuidas a lo largo de la Cordillera de la Costa (38°S to 41°S) y en la zona centro sur de Chile a través de la Cordillera de los Andes (36°S to 40°S). El objetivo de este estudio fue complementar las inferencias genéticas previamente determinada por isoenzimas, para obtener estimaciones mas adecuadas de la diversidad genética. A partir de las 33 bandas RAPD analizadas se observó un promedio de 88,1% de loci polimórficos con valores que fluctuaron entre 33% y 63%. El análisis análisis de varianza molecular (AMOVA) reveló que la mayor proporción de la variación genética se encuentra distribuída dentro de las poblaciones estudiadas (87,6%). Sin embargo, el valor de F_{ST} ($F_{ST} = 0.124$; $p < 0.00001$) indicó que hay una significativa diferenciación entre poblaciones. El análisis discriminante mostró la existencia de tres grupos geográficos definidos con 14 loci explicando el 87,2% de la diferenciación genética entre poblaciones. La prueba de neutralidad de Watterson y el análisis de desequilibrio de ligamiento (LD) de Ohta sugieren que la variación detectada podría ser explicada en parte por factores estocásticos demográficos y ambientales. El rol de las últimas glaciaciones así como algunas medidas de conservación y mejoramiento es discutido.

Palabras clave: Estructura genética, *Nothofagus*, marcadores RAPD.

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