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Primer Note: A New Set of Highly Polymorphic Nuclear Microsatellite Markers for *Nothofagus nervosa* and Related South American Species

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Abstract

Nothofagus is the main component of southern South American temperate forests. Overexploitation in the past has led to the loss of 40% of the original distribution range. Genetic diversity as well as biological processes shaping the distribution of the genetic variation (e.g. gene flow) constitutes basic knowledge for the implementation of conservation measures and for the definition of Evolutionary Significant Units. Nuclear microsatellites are the marker of choice for gene flow and fine-scale genetic structure studies. We enlarged a

previous set of microsatellites (SSRs) for South American *Nothofagus* species, with special concern to *Nothofagus nervosa* (Phil.) Dim. et Mil. Five new SSRs are presented with allele numbers up to 12 in a single population. The primers transferred well to five related species (*N. obliqua* (Mirb.) Oerst., *N. glauca* (Phil.) Krasser, *N. dombeyi* (Mirb.) Oerst., *N. pumilio* (Poepp et Endl.) Krasser and *N. antarctica* (G. Forster) Oerst., with allele numbers up to 11. The high level of polymorphism promises a sufficient power for gene flow and parentage analyses.

Key words: *Nothofagus*, microsatellites, South America, gene flow, fine-scale genetic structure.

Introduction

Nothofagus is the dominant genus of southern South American temperate forests, with a total of ten endemic species. In the last century, over-exploitation, over

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grassing, recurrent forest fires and agricultural settlement provoked the loss of 40% of the original distribution range (LARA et al., 1999). Conservation and domestication programs for maintaining the genetic resources and genetic diversity of some of the species have begun both in Chile and Argentina. Genetic studies have been conducted in some of the species with genetic markers such as isozymes (e.g. PREMOLI, 1997; MARCHELLI and GALLO, 2001; DONOSO et al., 2004), chloroplast DNA (MARCHELLI and GALLO, 2006), ISSR and RAPDs (MATTIONI et al., 2002). However, for gene flow studies and fine-scale genetic diversity analyses highly polymorphic nuclear microsatellites (SSRs) are the marker of choice. Microsatellites have been developed for Australian *Nothofagus* (JONES et al., 2004). Besides, transference of *Quercus* SSRs and the development of three nuclear microsatellites for South American *Nothofagus* were reported (MARCHELLI and GALLO, 2000; AZPILICUETA et al., 2004). Notwithstanding, additional loci are needed since among the three markers developed for South American species the number of alleles was lower than expected and amount of polymorphism was not enough for parentage analyses.

Here we present the development of an enlarged set of microsatellite markers for *Nothofagus nervosa* (Phil.) Dim. et Mil. (= *N. alpina* (Poepp. et Endl.) Oerst) for their use in gene flow and fine-scale genetic structure studies. Additionally, Mendelian inheritance was evaluated by analyzing the segregation of alleles among mothers and half sibs. The cross-amplification in five related species was also tested.

Materials and Methods

A microsatellite library was developed in *Nothofagus nervosa* using the microsatellite enrichment protocol (EDWARDS et al., 1996) and selective hybridization with the (AC)_n and (AG)_n insert repeats. PCR products were cloned using the Topo TA cloning Kit (Invitrogen) and sequenced on a Megabace1000 DNA analysis system using the cycle sequencing kit (Amersham Biosciences) according to manufacturer's instructions. Suitable sequences were selected and used to design PCR primers using the freely distributed software *Primer 3* on the WWW (ROZEN and SKALETSKY, 2000).

Table 1. – Characterization of five *Nothofagus nervosa* SSR loci.

Locus	Primer sequence	Repeat	T _a (°C)	MgCl ₂ concentration	Size range (bp)	A	A _{off}	H _o	H _e	GenBank accession no.
NnBIO 10	F: GATCCACATGTCCTTGGTC R: TGGACCGATAAGAATGTCAGC	(CA) ₁₇	53	1.5	262-292	8	12	1.0*	0.803	AM408112
NnBIO 111	F: TATGTGAACGCGTCTGCTTC R: CGCTCTTCAGACCAGAAAGG	(GT) ₂ A (GT) ₁₀	53	2.0	121-137	6	9	0.625	0.604	AM408113
NnBIO 37	F: CAACGACTGTTCCTCACTCC R: TTACCTGGTGGGAGATTTCG	(CT) ₁₂	59	1.5	111-135	8	10	1.0*	0.834	AM408114
NnBIO 72	F: TAAGTGGGGTGGTTTCCAAC R: ATTCCCATCTACCCTGAC	(TTC) ₇	54	2.5	149-179	7	11	0.700	0.768	AM408115
NnBIO 90	F: TCCCCTATTCTTGACCAACC R: TGGTTTTATTGGTGGAAATGG	(GT) ₄ G(GT) ₂ GC(GT) ₂	57	1.5	401	1	1	0	0	AM408116

T_a: Annealing temperature, H_o and H_e: observed and expected heterozygosities, A: allele number, A_{off}: allele number including offspring. * Significant heterozygote excess (P < 0.05).

To characterize the isolated microsatellites, buds from 25 *Nothofagus nervosa* trees from Tromen Lake in Argentina were collected. To test Mendelian segregation, 20 progenies from each of twelve of the mother trees obtained as open-pollinated seed were sampled. DNA was isolated following the procedure by DUMOLIN et al. (1995) with slight modifications according to MARCHELLI et al. (1998). PCRs were performed in a total volume of 20 µl containing 10 x PCR buffer (containing 200 mM Tris-HCl pH 8.4; 500 mM KCl; final concentration 1 x), between 1.5 to 2.5 mM MgCl₂ (Table 1), 0.1 mM dNTPs, 0.2 µM of each primer, 0.6% BSA, 1 U Taq polymerase (Invitrogen) and 25 ng of template DNA. Reactions were performed using a MJ Research PT-200 thermo cycler with the following profile: a hot start of 95°C for 5 min., followed by 35 cycles of denaturing at 94°C for 1 min., annealing temperature (Table 1) for 1 min, and extension at 72°C for 1 min., with a final extension at 72°C for 7 min. PCR products were denatured by the addition of 95% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol and 0.5M EDTA pH = 8 and heating at 94°C for 6 minutes. Denatured PCR products were run in 6% polyacrylamide sequencing gels containing 7 M urea in 1 x TBE buffer. Gels were silver-stained following the protocol of STREIFF et al. (1998) and the image scanned. For the determination of allele size, different genotypes were selected and run in an ABI 3100 sequencer (Applied Biosystems). Observed and expected heterozygosities, deviations from Hardy-Weinberg expectations and linkage disequilibrium among loci were calculated using GENEPOP version 3.4 (RAYMOND and ROUSSET, 1995). These analyses were done with the population samples, not including the offspring.

Cross-amplification was examined in five South American *Nothofagus* species: *Nothofagus obliqua* (Mirb.) Oerst, *N. dombeyi* (Mirb.) Oerst, *N. pumilio* (Poepp et Endl.) Krasser and *N. antarctica* (G. Forster) Oerst (15 individuals each) and *N. glauca* (Phil.) Krasser, (9 individuals).

Results and Discussion

Six microsatellites gave a good amplification product with scorable bands. The others showed no amplification or multiple band patterns. Among these six SSR loci,

Table 2. – Number of alleles of five SSR loci in *Nothofagus nervosa* and related *Nothofagus* species.

Species	N	NnBIO 10	NnBIO 111	NnBIO 37	NnBIO 72	NnBIO 90
<i>N. nervosa</i>	25	8	6	8	7	1
<i>N. obliqua</i>	15	11	8	5	5	2
<i>N. glauca</i>	9	4	1	6	6	3
<i>N. antarctica</i>	15	6	6	7	4	1
<i>N. pumilio</i>	15	6	5	5	3	1
<i>N. dombeyi</i>	15	6	4	8	6	1

N: number of individuals tested. Annealing temperatures are the same as in *N. nervosa*, except for NnBIO37 in *N. antarctica*, *N. pumilio* and *N. dombeyi* which is 55 °C.

one was monomorphic in *N. nervosa* and all related species, and therefore discarded from further analyses. For the remaining microsatellites, the number of alleles observed ranged from six to eight among the adult trees, but was higher when including the offspring reaching values of up to 12 alleles in NnBIO10 (Table 1). A particular case is locus NnBIO90 which was monomorphic in most of the species, but presented two and three alleles in *N. obliqua* and *N. glauca* respectively. It was monomorphic in the analysed samples of *N. nervosa*, and therefore not suitable as a microsatellite marker for gene flow studies. However, the presence of different alleles among *N. nervosa* and *N. obliqua* suggests the potential use of this locus as a diagnostic marker in studies of the natural hybridization between these two species. Segregation of alleles was proved by analyzing the maternal genotype together with the seedlings. Observed heterozygocities ranged between 0.625 and 1.0, while expected values were between 0.604 and 0.835 (Table 1). Significant heterozygote excess was observed for loci NnBIO10 and NnBIO37 ($P < 0.05$). The Fisher's test did not reveal any significant case of linkage disequilibrium.

Cross-amplification was successful in all the related species for the five primers and allele number ranged from one to 11 (Table 2). The allele sizes were more similar between species belonging to the same taxonomic clade. According to the phylogeny presented by Manos (1997) *N. nervosa*, *N. obliqua* and *N. glauca* form one taxonomic subgroup within the subgenera Lophozonia, while *N. antarctica*, *N. dombeyi* and *N. pumilio* belong to the subgenera *Nothofagus*. Within each subgroup most of the species hybridize naturally (e.g. GALLO et al., 1997; STECONNI et al., 2004). The existence of species-specific alleles for studying the hybridization was observed between *N. nervosa* and *N. obliqua* (data not shown) and the possibility of finding specific alleles for the other species is open.

The levels of polymorphism detected in *N. nervosa* with the SSRs described in the present work is very promising for their application to gene flow and fine-scale diversity studies. Furthermore, since sampling was done within one population an enlarged number of alleles are expected when extending the range. At present, the described microsatellites are being used for pollen flow studies and fine-scale structure of genetic diversity. Preliminary data from one population indicates that the exclusion probability is higher than 90% (unpublished data).

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Genetic Variation in the Qinghai-Tibetan Plateau Endemic and Endangered Conifer *Cupressus gigantea*, Detected Using RAPD and ISSR Markers

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Abstract

Assessing the level and distribution of genetic diversity of rare tree species is essential for their management and the development of effective conservation strategies. *Cupressus gigantea* is a long-lived endemic cypress of the west Qinghai-Tibetan Plateau and the tallest tree in its genus. The current populations of this species are fragmented and highly disturbed. We used RAPD (Random Amplified Polymorphic DNA) and ISSR (Inter-Simple Sequence Repeat amplification) markers to assess the genetic variation and population structure of this

endangered cypress. The 15 RAPD primers used in this study amplified 108 reproducible bands, 49 (45.4%) of which were polymorphic, while the 12 ISSR primers amplified 94 bands, 65 (69.2%) of which were polymorphic. Analysis of Molecular Variance (AMOVA) indicated that 49.7% and 38.3% of the variation was attributable to differences between populations for the RAPD and ISSR markers, respectively; relatively high compared to values reported for other conifer species. These estimates were also similar to *Gst* values obtained from Nei's gene diversity analyses (RAPD = 0.41 and ISSR = 0.36), and suggest that there is a high degree of population differentiation in this narrowly-distributed conifer. The genetic structure of this species has probably been shaped by its long life cycle and climatic changes during the Quaternary. The high degree of population differentiation in this species highlights the need for additional conservation measures, including measures to protect all of the remaining populations. The substantial similarities between the results of the RAPD and ISSR analyses of samples from the same individuals indicate that they can be interpreted with high levels of confidence.

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