SSR Markers for Analysing South American *Nothofagus* Species

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Summary

11 newly discovered microsatellites were used to identify SSR markers for characterising South American *Nothofagus* species. This was carried out in six species. The sample sizes used were between four and six individuals per species. The cross-genera transferability of 34 *Quercus* SSRs was also essayed. Out of the 11 new microsatellite markers, three proved to be polymorphic (NnBIO 11, NgBIO 13 and NgBIO 14). The qualitative confirmation of the inheritance of these markers could also be verified. Polymorphism was also observed in five of the cross-genera transferred SSRs (QrBIO7, quru-GA-0A01, quru-GA-0C11, quru-GA-0I01, quru-GA-0M07). The number of alleles per locus found range between 1 and 6 per species. The eight polymorphic SSRs identified in this study will constitute a valuable tool in the gene flow studies that are currently being carried out in natural populations of South American *Nothofagus* species. The confirmation of cross-species and cross-genera transferability opens the way for the use of SSRs as bridge markers in genetic mapping.

Key words: microsatellites, *Nothofagus*, transferability, *Quercus*.

Introduction

The genus *Nothofagus* is the principal component of Southern South American temperate forests, occurring on both sides of the Andes Mountains, in Chile and Argentina, with a wide latitudinal distribution from 33°S (central Chile) to 56°S in Tierra del Fuego, Argentina (VEBLEN *et al.,* 1996). This genus has been included for a long time within the *Fagaceae* family, although MANOS (1997) has proposed the term *Nothofagaceae* as the correct one. Twelve American taxa comprise this genus (VAZQUEZ and RODRIGUEZ, 1999) and some hybridise naturally when occurring sympatrically. Due to their high wood quality, some of these species were selectively exploited during the past and this has led to the actual situation that stands probably became genetically impoverished. Both *N. nervosa* and *N. obliqua* are the species that have mainly suffered from such an overexploitation.

Several studies have been carried out on South American *Nothofagus* forests in order to characterise their genetic variation. These studies were done by morphological as well as by ecological characters (DONOSO, 1979; MARCHELLI and GALLO, 1999), isozyme markers (PREMOLI, 1997; MARCHELLI and GALLO, 2001), combining morphological and isozymic characters (GALLO *et al.,* 1997), cpDNA markers (MARCHELLI *et al.,* 1998), ISSR and RAPDs (MATTIONI *et al.,* 2002). The natural hybridisation that occurred between some of the species and its impact on the genetic structure has been also studied. For example, hybridisation between *N. nervosa* and *N. obliqua*, first reported in plants on an arboretum in Britain (STEWART, 1979; TULEY, 1980) was further investigated by GALLO (2002). However, gene flow and paternity tests could not be done until now, due to the lack of more informative markers.

Microsatellites also named SSRs (simple sequence repeats) markers are short, tandemly repeated nucleotide motifs (1–6 bp) of the genome. They are abundant and highly polymorphic. These characteristics make SSRs powerful and highly discriminant markers for performing genetic studies within and among tree populations. Microsatellite primers have been designed for numerous woody plants including gymnosperms (e.g. RAJORA *et al.,* 2001; HODGETTS *et al.,* 2001; MORIGUCHI *et al.,* 2003), and angiosperms of the northern Hemisphere (e.g. DOW *et al.,* 1995; STEINKELLNER *et al.,* 1997; KAMPFER *et al.,* 1998; VAN DER SCHOOT *et al.*, 2000; ALDRICH *et al.,* 2002; ALDRICH *et al.,* 2003) and the southern Hemisphere (e.g. BRONDANI *et al.,* 1998; COLEVATTI *et al.,* 2001; BUTCHER *et al.,* 2000). Microsatellites have been recently reported for the Australian tree species *Nothofagus cunninghamii* (Hook) Oersted (JONES *et al.,* 2004). However, because cloning and characterisation of SSRs is expensive and laborious, they have not been found in case of South American *Nothofagus* species until now.

The aim of this study is to design and characterise SSR markers for American *Nothofagus* species. In order to enlarge this small number of genetic markers also SSRs from European and American oak species were applied to study genetic variation within South American tree species, based on the characteristic of transferability of microsatellites among related genera.

Materials and Methods

Genomic libraries, enriched by the repeat motifs CT/GA, TG/AC and GAC/CTG, were constructed for *N. nervosa*, *N. obliqua*, *N. dombeyi* and *N. glauca* following the procedures of EDWARDS *et al.* (1996). Modifications were carried out according to BUTCHER *et al.* (2000), by using an additional round of hybridisation. PCR products were subcloned using the Topo TA cloning Kit (Invitrogen) according to manufacturer instructions. For each tree species, 24 genomic clones were randomly chosen and sequenced by a Megabase1000 DNA analysis instrument using the cycle sequencing kit (Amersham Biosciences). Repeat motifs were identified in more than 50% of the sequenced clones and flanking nucleotides were selected in order to design PCR primers by means of a share-ware programme Primer 3 (ROZEN and SKALETSKY, 2000). All SSR primers tested for *Nothofagus* spp. are shown in *Table 1.*

In addition, the following PCR primers originally designed for *Quercus robur* L. and *Q. petraea* (Matt.) Liebl. were tested: QrZAG2, QrZAG15, QrZAG58, QrZAG90 (KAMPFER *et al.,* 1998); QrBIO7, QrBIO 8, QrBIO 9, QrBIO 10, QrBIO 11, QrBIO 12, QrBIO 22, QrBIO 24, QrBIO 25, QrBIO 26, QrBIO SA, QrBIO SB (Caron, personal communication), QpZAG1/2, QpZAG110 (STEINKELLNER *et al.,* 1997). Moreover, some SSR primers reported for *Quercus rubra* L. were tested using a Licor DNA Sequencer 4000 instrument, namely quru-GA-1C06, quru-GA-1G13, quru-GA-0C11, quru-GA-0E09, quru-GA-0I01, quru-GA-0M05, quru-GA-0A01, quru-GA-0C19, quru-GA-0M07, quru-GA-1F02, quru-GA-1C08, quru-GA-2F05, quru-GA-2M04 described by ALDRICH *et al.* (2002) as well as quru-

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Table 1. – New set of SSR identified for *Nothofagus* species. Species from which they were isolated, primer sequence, repeated motifs, annealing temperature (Ta) and results of amplification and polymorphism are detailed.

						PCR results in different species tested					
Code	**Species**	**Primer sequences**	**Repeat motifs**	**N**	**Ta**	***N.n***	***N.o***	***N.p***	***N.a***	***N.d***	***N.g***
NdBIO2	*N. dombeyi*	F: AAGGGGAATACTTAGGCAAC R: CATTGGAGACTTTCAAGGAG	$(TG)_{10}$	4	55	M	M	M	M	M	M
NdBIO 6	*N. dombeyi*	F: CTAGGACCTGAATTATTGGC R: TCTGATTTCTCTCAAAAGGG	$(CA)_{10}$	4	55	M	M	M	M	M	M
NdBIO 13	*N. dombeyi*	F: TAAATCAACAAAGGGTCAGC R: CAGATGGTTGAGACTTGAGAG	$(GT)_{12}$	4	55	+	+	-	-	+	+
NdBIO 16	*N. dombeyi*	F : AATAAAACAAGCAAGGCAAG R: GCCAATTGTTTGTGAGGTAG	$(GA)_{12}$	4	55	I	I	I	I	I	I
NoBIO 2	*N. obliqua*	F: TTGAGACTTGAGAGAATCGC R: TAAATCAACAAAGGGTCAGC	$(CA)_{15}$	4	55	I	I	I	I	I	I
NnBIO 11	*N. nervosa*	F: AGGGACGCACATTTCAAAAC R: GACTTGGCCTACATCACTTCTTC	$(GT)_{10}$	6	59	P (3)	P (6)	-	-	-	-
NgBIO 7	*N. glauca*	F: GAACCACGTAAATCTTGTG R: CAAAACGATAAGAGGGAATC	$(TG)_{10}$	4	55	+	-	-	-	-	+
NgBIO 8	*N. glauca*	F: AGTGAGTGGTAATCCAGGTG R: ATGTGATGAGATGAGAAGCC	$(CA)_{12}$	4	55	M	M	M	M	M	M
NgBIO 12	*N. glauca*	F: TTTGTAACGACAATCATTCG R: AAGGGGAATACTTAGGCAAC	$(CA)_5CG(CA)_5$	4	55	M	M	M	M	M	M
NgBIO 13	*N. glauca*	F: GAGACGTTAGATGGGTTTTG R: AGACCAACCAGTTCAACAAG	$(CT)_{14}CC(GT)_8$	6	55	P (4)	P (4)	-	-	-	-
NgBIO 14	*N. glauca*	F: AGAACACAGACAGATCACCC R: TATTTTGGACATGATCCTTG	$(CA)_3(CACAA)_4(CA)_3$	5	55	P (2)	P (3)	+	M	-	-

References

Nn: N. nervosa – No: N. obliqua – Np: N. pumilio – Na: N. antarctica – Nd: N. dombeyi – Ng: N. glauca

M = monomorphic bands (n > 1); P = polymorphic (between brackets, the total number of alleles observed); I = irregular bands; –: no amplification, +: amplification (n = 1)

N = number of individuals analysed

GA-0A03, quru-GA-2G07 and quru-GA-1D09 reported by ALDRICH *et al.* (2003). For this purpose, the forward primers were labelled by a fluorescent dye.

Four to six individuals each of six *Nothofagus* test species [*N. nervosa* (Phil.) Dim. et Mil., *N. obliqua* (Mirb.) Oerst., *N. pumilio* (Poepp. et Endl.) Krasser, *N. antarctica* (Forst.) Oerst., *N. dombeyi* (Mirb.) Oerst., and *N. glauca* (Phil.) Krasser] were analysed. *N. glauca* was sampled in Chile, whereas the other species were collected in Argentina. Additionally, a preliminary qualitative segregation analysis was carried out with half-sib progenies (two in case of *N. obliqua* and two in case of *N. nervosa* with 15 individuals per progeny).

The DNA was extracted from buds, using the protocol reported by DUMOLIN *et al.* (1995), with slight modifications. The PCR reaction was carried out in a total volume of 20 µl containing 10X buffer, 1.5 mM MgCl, 0.1 mM dNTPs, 0.2 µM of each primer (forward and reverse), 0.8U Taq DNA polymerase (Invitrogen) and 10 ng DNA. Reactions were performed in a Perkin Elmer 2700 DNA thermal cycler. The programme used with the unlabelled primers consisted of a hot start of 94 °C for 5 min, followed by 35 cycles of denaturing at 94 °C during 45 seconds, annealing at 55 °C (59 °C for SSR NnBIO11) for 45 seconds and extension at 72 °C for 45 seconds, with a final extension at 72 °C for 7 minutes. The following touch-down programme was used for the labelled primers: 3 min denaturation at 94 °C; 2 cycles of 1 min denaturation at 94 °C, 1 min at 60 °C and 35s at 70 °C, followed by 18 cycles of 45s denaturation at 93 °C – 45s annealing at 50 – 59.5 °C and 1 min extension at 70 °C and finally 20 cycles of 30 s at 92 °C, 30 s annealing at 50 °C and 1 min extension at 70 °C with a final elongation at 70 °C for 20 min. The PCR products from unlabelled and labelled primers were denatured by the addition of a stop solution (95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol, 10 µM NaOH) heating for 6 minutes at 94 °C. Following denaturation the fragments amplified with the unlabelled primers were run in a 6% polyacrylamide sequencing gel containing 7 M of urea in 1x TBE buffer. The gel was silver-stained using the protocol published in STREIFF *et al.* (1998) and the image scanned. The fluorescent PCR products were run in a polyacrylamide gel containing 6 M of urea and 1x TBE buffer by using the Li-cor DNA Sequencer. Transferability between techniques (sequencer *vs.* silver nitrate staining) was also analysed in case of applying *Q. rubra* SSR motifs in laboratories not equipped with sequencing instruments.

Results

From the 11 new repeat motifs found in single *Nothofagus* spp. and tested in four or six individuals per species, only three confirmed polymorphic bands (NnBIO11, NgBIO13 and NgBIO14) for the species and primers indicated in *Table 1.* The estimated average fragment sizes for the motifs were 200 bp, 180 bp and 250 bp, respectively. The preliminary results of a segregation analysis (*Table 2*) confirmed the qualitative mode of inheritance of these markers.

From the European oak SSRs transferred in three *Nothofagus* species, the unlabelled marker QrBIO7 (primer sequence F:CAGACCACTCTCTCTTCTGC and R:GACTCACTATAGGCCGAATG) showed polymorphism (*Table 3*). Four labelled

Table 2. – Preliminary qualitative segregation analysis in half-sib families of *N. nervosa* and *N. obliqua.*

Primer	**Species/Family**	**N° individuals**	**NA**	**NA_T**
NgBIO 14	*N.obliqua* 18	11	3	3
NgBIO 13	*N.obliqua* 18	11	3	4
	N.obliqua 111	10	3	
NnBIO 11	*N.obliqua* 18	9	4	6
	N.obliqua 111	10	4	
	N.nervosa 8	9	3	

NA: number of alleles observed/family; NA_T = number of alleles observed/primer.

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Table 3. – Number of alleles per species found in the transferred *Quercus* primers.

	Number of Alleles		
Primer	***N. obliqua***	***N. nervosa***	***N. betuloides***
QrBIO7	3	-	-
quru-GAOC11	2	3	2
quru-GA0I01	4	2	2
quru-GA0M07	2	-	2
quru-GA0A01	2	-	2

Q. rubra primers namely, quru-GA-0A01, quru-GA-0M07, quru-GA-0I01 and quru-GA-0C11 also exhibited polymorphism at species level (*Table 3*) with ranges of fragment sizes of 109-133 bp, 111-117 bp, 175-234 bp and 197 bp, respectively.

The number of alleles per locus found in this study including the cross-species and cross-genera amplifications range between one and six (*Table 1*).

The transferability between techniques (labelled primers run in silver staining gels) resulted possibly in SSRs quru-GA-0A01, quru-GA-0C11 and quru-GA-0M07, although the annealing temperature had to be optimised for silver staining gels (data not shown).

Discussion

The number of alleles per locus found per species in the study (1–6) was lower than those reported in other tree species studies: 4–12 in *Nothofagus cunninghamii* (Jones *et al.,* 2004), 5–18 in *Eucalyptus urophylla* and *E. grandis* (Brondani *et al.,* 1998), 10–19 in *Populus nigra* (van der Schoot, 2000), 5–12 in *Malus x domestica* (Gianfranceschi *et al.,* 1998), 5–11 in *Populus tremuloides* (Dayanandan *et al.,* 1998) and higher than the one observed in *Prunus persica*, a self-fertile and self-pollinating fruit species, with 2–4 alleles per locus (Cipriani *et al.,* 1999). The low number of alleles observed in the genus *Nothofagus* could probably be associated with the relatively small population sample size used in this study (4–6 individuals per species and 15 per family). However, an estimated self-pollinating rate of 6%, as reported in *Nothofagus nervosa* (Gallo *et al.,* 1997), which reduces the genetic variation, could also be a factor influencing these results.

Cross-species amplification could successfully be carried out as reported in other studies (e.g. Aldrich *et al.,* 2003; van der Schoot *et al.,* 2000; Dayanandan *et al.,* 1998; Kampfer *et al.,* 1998; Cipriani *et al.,* 1999). Cross-amplification success ranged from absence to 100%; values ranging from 43% to 100% occurred in the Australian *Nothofagus* (Jones *et al.,* 2004). The number of repeat motifs in South American *Nothofagus* primers was 10 to 23 and 7 to 25 in the Australian one – with differences in the repetitive nucleotides. Transferability between related genera was also possible as four American and one European *Quercus* SSRs were successfully applied to *Nothofagus* genus. This reconfirms the possibility of SSRs' transferability between related genera reported in other studies (White and Powell, 1997; Yamamoto *et al.,* 2001; Dirlewanger *et al.,* 2002; Barreneche *et al.,* 2004; Aldrich *et al.,* 2003). However, this transferability failed in the rest of the SSRs tested as also occurred in other studies in which inter-genera transferability was not possible (e.g. Butcher *et al.,* 2000). From the four polymorphic SSRs' *Quercus rubra* found in *Nothofagus obliqua* and *N. nervosa*, quru-GA0A01, quru-GA0C11 and quru-GA0M07 also showed amplification in European *Quercus* and *Castanea genera* (Barreneche *et al.,* 2004; Aldrich *et al.,* 2003). The SSR quru-GA0A01 exhibited the same number of alleles showed in the species for which it has been developed. SSR quru-GA0M07 primer amplified products with lower size fragments according to the *Q. rubra* study. Two of the polymorphic primers showed size fragments similar to those in *Quercus rubra*, with slight differences.

The success of transferability depending on the evolutionary level of relatedness of the taxa being sampled was difficult to confirm in this study since we verified high level of positive amplification in both cross-species and cross-genera amplification. This also occurred when applied peach microsatellites to other species, even those belonging to different families also showing a good level of amplification (Dirlewanger *et al.,* 2002).

The eight polymorphic SSRs identified in this study will be a valuable tool for the gene flow studies that are being carried out at this moment in pure and hybrid populations of *Nothofagus* species. The hyper-variability and co-dominance mode of inheritance constitute great advantages in SSRs for these types of studies (e.g. Lexer *et al.,* 1999; Colevatti *et al.,* 2001). Additionally, the possibility of SSR transferability reported in this study could constitute a strategic tool for those species for which SSRs have not been developed yet. On the other hand, given this possibility, an additional value is obtained because of the importance of having common markers between species/genera to be used as bridge markers in genetic mapping.

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