Isolation and characterization of microsatellites for the endangered endemic tree *Nothofagus alessandrii* (Nothofagaceae)

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**Abstract**

*Nothofagus alessandrii* (Nothofagaceae) is one of the most endangered trees from Chile due to high rates of habitat disturbance caused by human activities. Despite its conservation status, few molecular markers are available to study its population genetic, connectivity and to assist reproduction programs. Thus, the species needs urgent actions to restore its original distribution. Novel polymorphic microsatellites from the genome of *N. alessandrii* were isolated and characterized using high-through sequencing. A total of 30 primer pairs were synthesized and 18 microsatellites were amplified correctly. Polymorphism and genetic diversity was evaluated in 58 individuals from three populations of *N. alessandrii*. Sixteen of them were polymorphic and the number of alleles in the pooled sample ranged from 2 to 14, the mean number of alleles was 4.81. The mean values of observed heterozygosity (*H*o) and excepted heterozygosity (*H*e) are similar in all studied populations. Linkage disequilibrium was found between a few pairs of loci (five out of 263 tests) suggesting that most of the markers can be considered as independent. Significant deviations from Hardy–Weinberg equilibrium (*P* < 0.05) were found in four loci probably due to low sampling size. Transferability to the congeneric *N. pumilio* was successful in only four out of the sixteen polymorphic markers. The microsatellite markers developed in this study will be useful to study the genetic diversity and structure and to develop integrated management plans for the conservation of this endangered species.

**Keywords** *Nothofagus alessandrii* · Conservation genetics · Endangered species · Nothofagaceae · Next-generation sequencing

**Introduction**

*Nothofagus alessandrii* (Nothofagaceae), also known as “ruil”, is a broad-leaf deciduous tree endemic to the Mediterranean climate zone of central Chile (35-36ºS). The species is among the most primitive species within the genus [1, 2] and has been considered as a “living fossil” [3]. The International Union for Conservation of Nature [IUCN] classified *N. alessandrii* as endangered in 1997 [4], status that is maintained nowadays [5]. Currently, the species is restricted to a restricted latitudinal range (116 km) having a narrow area of occupancy (755 km²). From the last decade of the XIX century, the forest dominated by *N. alessandrii* became increasingly reduced and fragmented. Firstly, the species was overexploited for wood (posts and poles) being afterwards replaced by plantations of wheat, and more recently, by *Pinus radiata* and *Eucalyptus globulus*. In addition, in the last decades the species has been threatened by anthropogenic origin forest fires, which have increased their intensity and frequency [6]. Consequently, the remnant populations of *N. alessandrii* represent a small and highly fragmented sample of a more extended distribution [7], where most of its stands are secondary growth from stumps sprouts. In addition to those threats, the species shows low reproductive rates (i.e., low seed production and low seedling survival [8, 9]), which are likely to be the result of inbreeding due...
reduced population sizes and restricted gene flow caused by anthropogenic deforestation.

Microsatellite (short sequence repeats, SSRs) markers have been widely used in conservation genetics. For instance, SSRs have been used to estimate the levels of within and among genetic diversity of endangered species [10], to delineate conservation units [11], to estimate inbreeding and gene flow [12], among others. This information is critical to design management strategies to restore endangered species [13, 14].

The aim of this study was to develop new microsatellite markers (simple sequence repeats SSRs) for *N. alessandrii*, using high-throughput Illumina sequencing platform. We also tested cross-amplification in one congener species (*N. pumilio*). These new markers should contribute study the genetic diversity of the species at different scales (i.e., fine-scale within population, within and among populations, landscape scale) and to develop conservation strategies based on molecular ecology studies.

**Material and methods**

**DNA extraction and genome sequencing**

To determine the polymorphism of microsatellites we collected plant material from three populations: (i) Los Ruiles National Reserve, sector “Chanco” (CHA, 35°50′S, 72°30′W), (ii) Los Ruiles National Reserve, sector “Empeñado” (EMP, 35°37′S, 72°20′W) and (iii) Fundo El Desprecio (FED: 35°40′ S, 72°18′W). The two former populations are the only protected areas for the species. Field samples were collected with permission from the Chilean Forestry Service (CONAF; Permission code: 05–2018). Young leaves from a total of 58 adult individuals of *N. alessandrii* were stored and dried in silica gel before DNA extractions. From each population, a branch of one individuals was taken. Given that *N. alessandrii* is phylogenetically distant to its South American congeners [1, 2], being closer to New Zealand species, we decided to test cross-amplification in *N. pumilio* only, as low transferability is the more likely result.

Genomic DNA (gDNA) from both species was extracted using DNeasy Plant mini Kit (Qiagen Valencia, CA, USA) from dried leaves following manufacturer protocol. All the DNA extracts were quantified and standardized using fluorometric method (Qubit® 3.0, Invitrogen). The purified genomic gDNA was normalized to 0.2 ng/µl and subsequently processed using Nextera® XT DNA library preparation following the manufacturer’s instructions. Paired-end high-throughput sequencing was performed on an Illumina HiSeq 2000 platform (Illumina, San Diego, CA, USA) by Australomics Valdivia, Chile (http://australomics.cl). The software QDD version v3.1 [16] was used to detect microsatellite motifs on reads longer than 80 pb. After this, BLASTn [17] was used to compare all sequences containing repeated motifs with at least 95% of similarity. This program identified 2,706 sequences containing SSRs associated to a single group and those that could not be assigned to a group (singletons). The design of forward and reverse primers was conducted with Primer3 v4.1.0 [18]. A subset of 39 putatively polymorphic SSR’s was obtained and a total of 30 primer pairs were synthesized for the subsequent polymorphism screening and genetic diversity estimates. Raw reads for the successfully amplified microsatellites were submitted to National Center for Biotechnology Information (NCBI), Sequence Read Archive (SRA): accession No. MW397624-MW387641 (Table 1). In order to facilitate other researchers to find more markers, either SSRs or single nucleotide polymorphisms (SNPs), we deposited raw sequencing data in the NCBI platform (BioSample: SAMN17980487, BioProject: PRJNA702808).

**PCR analyses and genotyping**

For the amplification test, PCRs were performed in a final volume of 10 µL containing: 1X PCR buffer, 1U of Taq DNA polimerase, 1.5 mM of MgCl₂, 0.12 mM of each dNTP and 10 µM of fluorescent forward and reverse primers. Forward primers were labeled with PET, FAM, VIC and NED fluorescent dyes (Applied Biosystems) in order to perform capillary sequencing in a genetic analyzer. The amplification protocol was as follows: an initial denaturation step at 95 °C for 5 min, 35 cycles consisting of a denaturation step at 95 °C for 30s, specific annealing temperature (Ta) of each primer pair for 30 s (Table 1), an extension step at 72 °C for 35 s, followed by a final step of DNA extension at 72 °C for 7 min. Each primer was amplified separately and then assigned to one of four mixes before capillary sequencing (Table 1). The final PCR products were analyzed on an ABI PRISM 310 Genetic Analyzer with GeneScan 500 LIZ (Applied Biosystems) at the DNA sequencing service of the Pontificia Universidad Católica de Chile (PUC), Santiago de Chile.
Microsatellite loci characterization

The software GENALEX v6.1 [19] was used to estimate the number of alleles per locus \((A)\), the effective number of alleles per population \((A_E)\), private alleles \((A_P)\), the observed heterozygosity \((H_O)\), expected heterozygosity \((H_E)\), inbreeding coefficient \((F)\), Hardy–Weinberg equilibrium (HWE) significance, and among populations genetic differentiation \((\theta_{ST})\). Linkage disequilibrium was tested using GENEPOP version 4.2 [20]. Null allele frequencies were assessed in MICRO-CHECKER v2.2.3 [21] using Brookfield’s estimator 1 [22]. The statistical significance was assessed using Bonferroni corrected \(P\) values.

### Table 1 Characteristics of 18 microsatellite markers developed for *Nothofagus alessandrii*

| Locus | Primer sequence (5'-3') | Repeat motif | Allele size (bp) | Fluorescent Dye | Mix* | Ta (°C) | GenBank accession no. |
|-------|--------------------------|--------------|------------------|-----------------|------|---------|----------------------|
| *Na01* | F: GCTTCGGGACCTATCAAGA | (AAG)\(_5\)  | 212              | VIC             | 1    | 60      | MW387624            |
|        | R: AGGGTGCGCTGTATCTACC   |              |                  |                 |      |         |                      |
| *Na02* | F: CGCTGACACCATAGATCGAG | (AG)\(_20\) | 254-266          | NED             | 2    | 59      | MW387625            |
|        | R: CCATATCCATAGAAGTGGCA  |              |                  |                 |      |         |                      |
| *Na04* | F: TGGGCTTGTGGAAGCTATGA | (AG)\(_3\)  | 125-127          | 6-FAM           | 3    | 59      | MW387626            |
|        | R: ACAGCTGTATAGCTTTCGC   |              |                  |                 |      |         |                      |
| *Na05* | F: ACATGCCCTGCAAGCATAT  | (AG)\(_3\)  | 288-290          | VIC             | 2    | 60      | MW387627            |
|        | R: GGAAGCAAATGCTAGTGTGA  |              |                  |                 |      |         |                      |
| *Na07* | F: GGGCCGACTTCTTGTTTACT | (AT)\(_16\) | 214-234          | 6-FAM           | 2    | 60      | MW387628            |
|        | R: TGTCAGACTGACCTGGTATTA |              |                  |                 |      |         |                      |
| *Na08* | F: GCACTTTCAACCCATACAGT | (AT)\(_3\)  | 234-236          | PET             | 3    | 60      | MW387629            |
|        | R: CAGGCTTTTGGATGAAATGCG |              |                  |                 |      |         |                      |
| *Na09* | F: CAAACAATGAGTACCAGATGA | (AT)\(_6\)  | 152-154          | VIC             | 1    | 60      | MW387630            |
|        | R: TGTAGGGAGAACGATGCTG   |              |                  |                 |      |         |                      |
| *Na10* | F: AATAAGGAGATCTCGGACAC | (AAT)\(_3\) | 136-145          | NED             | 4    | 59      | MW387631            |
|        | R: CCAAGGAACTATGGAATACACA |            |                  |                 |      |         |                      |
| *Na11* | F: TTCCATGACATGAGATGCC   | (AG)\(_14\) | 151-177          | PET             | 2    | 60      | MW387632            |
|        | R: CAAGCTCAACTCTCTCTCA   |              |                  |                 |      |         |                      |
| *Na12* | F: CAAGGTTGAGTACCTTCAAGC | (AG)\(_7\)  | 120-128          | VIC             | 2    | 58      | MW387633            |
|        | R: CCGTCCAAGCTGCTCAAATAC |              |                  |                 |      |         |                      |
| *Na13* | F: AAAGGACATTATAGACCTTATGGA | (AG)\(_3\)  | 102-110          | PET             | 1    | 57      | MW387634            |
|        | R: TACCCAAATATCTTATCTTTCATAT |            |                  |                 |      |         |                      |
| *Na14* | F: GCGTTCGCCATAAAATAACGA | (AAG)\(_8\) | 186              | 6-FAM           | 1    | 61      | MW387635            |
|        | R: AGACCCGAGAGACTCAGTGC  |              |                  |                 |      |         |                      |
| *Na16* | F: ACAGCCTAAGAACCCTCAACC | (AG)\(_15\) | 173-189          | PET             | 4    | 52      | MW387636            |
|        | R: CAACCCCTGACCTCAATGCC  |              |                  |                 |      |         |                      |
| *Na17* | F: CAAACATTTAACAACGAAAGCC | (AG)\(_14\) | 212-218          | VIC             | 4    | 51      | MW387637            |
|        | R: CCTATTTATAAAAATCCATCTTCG |            |                  |                 |      |         |                      |
| *Na23* | F: CCGTATGAGGAGTGAACCCG  | (TC)\(_15\) | 235-239          | NED             | 1    | 52      | MW387638            |
|        | R: GCATGGAAAGAACAAATACATCC |            |                  |                 |      |         |                      |
| *Na26* | F: CCGCCCATTTCTACTTGACC | (TC)\(_14\) | 278-288          | VIC             | 3    | 55      | MW387639            |
|        | R: GGAACTTACCGGGCAACCG   |              |                  |                 |      |         |                      |
| *Na27* | F: TTCCTAGCAAACAAAACTCTAGATGG | (AG)\(_8\)  | 301-303          | NED             | 3    | 53      | MW387640            |
|        | R: AATCCGGTGCTTACAGGTTCC |              |                  |                 |      |         |                      |
| *Na28* | F: AAGTTCTCTCTGTCTACCCGTG | (AT)\(_13\) | 315-319          | PET             | 4    | 51      | MW387641            |
|        | R: TGAAAGCGCTTGAATTAGGACC |              |                  |                 |      |         |                      |

The two markers highlighted in grey (*Na01* and *Na14*) were monomorphic across all populations

*PCR reactions were carried out separately and then assigned to one of four mixes before capillary sequencing analyses*
Results and discussion

A total of 41,144 reads with an average length of 392 bases were obtained from the shotgun sequencing. A total of 2,706 microsatellites were identified in the dataset (6.5% of total abundance). Primer pairs were synthesized for 30 out of the 39 putative polymorphic microsatellites. Eighteen microsatellites, ranging from 106 to 319 bp, amplified correctly (Table 1), and sixteen of them were polymorphic (Table 2), including one tri-nucleotide and fifteen di-nucleotide repeat markers. Considering all populations, a total of 77 alleles were scored. The number of alleles per locus ranged from 2 to 14, with a mean of 4.81 alleles per locus. The mean numbers of effective alleles per locus ($A_E$) were 2.10, 1.82, and 2.06 for CHA, EMP and FED, respectively (Table 2). Eleven, two and five private alleles ($A_P$) were found in CHA, EMP and FED, respectively. For the entire dataset, the expected ($H_O$) and observed ($H_E$) heterozygosities ranged from 0.000 to 0.856 and from 0.000 to 1.000, respectively. The mean $H_O$ and $H_E$ values in CHA population were 0.417 and 0.406, respectively. Similar values were found in populations EMP ($H_O=0.422, H_E=0.383$) and FED ($H_O=0.422, H_E=0.420$). Those values were lower to those of the restricted $N$. glauca ($H_O=0.457, H_E=0.502$; [23]) and $N$. macrocarpa ($H_O=0.405, H_E=0.625$; [24]) but lower than those of the more widely distributed $N$. obliqua ($H_O=0.600, H_E=0.662$; [23]) and $N$. alpina ($H_O=0.620, H_E=0.617$; [23]). Among-population genetic differentiation of $N$. alessandrii was high ($R_{ST}=0.251$) compared to their narrow congeners $N$. glauca ($R_{ST}=0.087$) and $N$. macrocarpa ($R_{ST}=0.084$) and their widely distributed congeners $N$. obliqua ($R_{ST}=0.110$) and $N$. alpina ($R_{ST}=0.160$) [23, 24]; however, our estimation of $R_{ST}$ should be seen with caution because we sampled only three populations and 18–20 individuals per population.

Considering the entire gene pool, significant deviations ($P<0.05$) from HWE were detected in six out of the sixteen loci ($Na02$, $Na04$, $Na07$, $Na08$, $Na13$ and $Na16$), possibly due to the small sampling size but also due homozygous and heterozygous excess. Null alleles were detected in only three loci from CHA ($Na02$, $Na12$, and $Na16$) and two loci from FED ($Na11$, $Na12$). In general, few loci showed evidence of linkage disequilibrium (LD). Considering the entire dataset only two loci, $Na17$ and $Na28$, were significantly linked ($Chi^2=17.6, P=0.024$). Within populations, LD was detected between two pairs of loci in CHA ($Na02$–$Na09$, $P=0.039$; $Na04$–$Na07$, $P=0.024$) and FED ($Na16$–$Na17$, $P=0.042$; $Na17$–$Na28$, $P=0.048$). In EMP population, LD was detected between only one pair of loci ($Na16$–$Na28$, $P=0.013$). Hence, we consider that the microsatellites developed in this study are mostly independent markers.

Overall, from the 18 markers developed here, two loci were monomorphic ($Na01$ and $Na14$), five showed low number of alleles ($Na04$, $Na05$, $Na08$, $Na09$ and $Na27$), and some showed null alleles. Nonetheless, the other eleven markers are polymorphic and therefore useful for population genetic studies. Despite this, researchers have to be careful when selecting loci for future studies.

As predicted, cross-amplification in $N$. pumilio was low, with successful amplification at only four out of the sixteen tested loci ($Na04$, $Na09$, $Na10$ and $Na12$). While $Na04$ was monomorphic, $Na09$, $Na10$ and $Na12$ had two (150 and 164 bp), two (139 and 142 bp) and four (120, 128, 130 and 134 bp) alleles, respectively.

To date, only 12% (0.42 km$^2$) of the distributional range of $N$. alessandrii is under protection in the National System of Protected Areas (SNASPE) of Chile and only two populations are considered within these protected areas: Los Ríos National Reserve Empedrado (EMP) with 0.25 km$^2$ and Chanco (CHA) with 0.45 km$^2$ (both studied here). Consequently, most of the species range of distribution is unprotected but also exposed to an increased frequency of anthropogenic fires. Currently, only one study [25] has addressed the genetic structure of the species but at a very limited extent (only seven fragments) and using allozymes. The 16 microsatellites developed for $N$. alessandrii can be used to assess a wide variety of questions related to conservation genetics such genetic structure, gene flow, inbreeding, definition of priority conservation areas but also to address the potential effects of habitat fragmentation on genetic diversity and reproduction in this endangered species. Habitat fragmentation generally has negative impacts on genetic diversity [26], reproductive success [27] and progeny quality [28]. Those markers can also help to evaluate if those negative impacts of habitat fragmentation have affected this endangered species. Although, habitat loss and fragmentation has drastically reduced the species distribution, almost all remaining trees are re-sprouts from cut and burned individuals. Considering that habitat fragmentation is recent compared to its longevity, the species should retain a significant proportion of its original genetic diversity [25]. Our results would help to obtain genetically based knowledge that is urgently required to support adequate conservation programs for this endangered species.
Table 2 Genetic diversity estimates for three populations of the endangered tree *Nothofagus alessandrii* in central Chile

| Locus | CHA (N = 18) | EMP (N = 20) | FED (N = 20) |
|-------|--------------|--------------|--------------|
|       | AT           | A            | AE           | Null         | A            | AE           | Null         | A            | Null         | A            | Null         | A            | Null         | A            | Null         | A            | Null         |
| Na02  | 7            | 4            | 2.23         | 0.2436*      | 5            | 2.39         | 0.0474       | 0.450        | 0.581        | 0.226**      | 1.167         | 0.551        | 0.697*       | 4            | 2.93         | 0.1066       | 0.450        | 0.699        | 0.317*       |
| Na04  | 2            | 2            | 1.95         | −0.2284      | 2            | 2.00         | −0.2632       | 0.900        | 0.500        | −0.380***     | 0.833         | 0.486        | −0.714**     | 2            | 2.00         | −0.3333       | 1.000        | 0.500        | −1.000***    |
| Na05  | 2            | 1            | 1.00         | 0.0000       | 1            | 1.00         | 0.0000       | 0.000        | 0.000        | NA           | 0.000        | 0.000        | NA           | 2            | 1.05         | −0.0013       | 0.050        | 0.049        | −0.026       |
| Na07  | 9            | 7            | 2.91         | −1.1675       | 7            | 3.60         | −0.0649       | 0.850        | 0.723        | 0.176        | 0.944         | 0.656        | −0.440***     | 5            | 2.52         | −0.1466       | 0.850        | 0.603        | −0.411***    |
| Na08  | 2            | 2            | 1.99         | −0.2956      | 2            | 2.00         | −0.2292       | 0.800        | 0.500        | −0.600**      | 0.944         | 0.498        | −0.895***     | 2            | 2.00         | −0.3333       | 1.000        | 0.500        | −1.000***    |
| Na09  | 2            | 2            | 1.53         | −0.0814      | 2            | 1.05         | −0.0013       | 0.050        | 0.049        | −0.026       | 0.444         | 0.346        | −0.286       | 2            | 1.11         | 0.0907        | 0.000        | 0.095        | 1.000***     |
| Na10  | 4            | 2            | 1.86         | −0.0903      | 4            | 2.32         | 0.0483       | 0.500        | 0.569        | 0.121        | 0.611         | 0.461        | −0.324       | 2            | 1.96         | 0.0843        | 0.350        | 0.489        | 0.284        |
| Na11  | 14           | 10           | 6.97         | 0.0458       | 10           | 4.94         | 0.1765*       | 0.500        | 0.798        | 0.373**      | 0.722         | 0.856        | 0.157        | 6            | 2.19         | 0.0757        | 0.450        | 0.544        | 0.172**      |
| Na12  | 3            | 3            | 1.33         | 0.1582*      | 2            | 1.41         | 0.1906*       | 0.050        | 0.289        | 0.827***     | 0.056         | 0.245        | 0.774***     | 2            | 1.11         | 0.0907        | 0.000        | 0.095        | 1.000***     |
| Na13  | 5             | 3            | 1.33         | 0.0672       | 2            | 1.34         | −0.0282       | 0.300        | 0.255        | −0.176       | 0.167         | 0.248        | 0.329***     | 2            | 1.28         | −0.0186       | 0.250        | 0.219        | 0.523        |
| Na16  | 9             | 4            | 2.14         | 0.1868*      | 3            | 2.19         | 0.0823       | 0.400        | 0.545        | 0.266***     | 0.278         | 0.532        | 0.478***     | 6            | 3.04         | 0.0223        | 0.600        | 0.671        | 0.106*       |
| Na17  | 4             | 4            | 2.17         | −0.0315      | 2            | 1.88         | −0.1853       | 0.750        | 0.469        | −0.600***     | 0.611         | 0.539        | −0.135       | 2            | 1.72         | 0.1050        | 0.300        | 0.420        | 0.286        |
| Na23  | 3             | 2            | 1.98         | 0.0903       | 2            | 1.92         | 0.1155       | 0.300        | 0.480        | 0.375        | 0.444         | 0.494        | 0.100       | 2            | 1.60         | −0.0051       | 0.400        | 0.375        | −0.067       |
| Na26  | 6             | 1            | 1.00         | 0.0000       | 4            | 1.67         | −0.0133       | 0.400        | 0.404        | 0.000        | 0.000        | 0.000        | 0.000        | NA           | 4            | 1.14         | −0.0367       | 0.350        | 0.306        | −0.143       |
| Na27  | 2             | 2            | 1.06         | −0.0149      | 1            | 1.00         | 0.0000       | 0.000        | 0.000        | NA           | 0.056        | 0.054        | −0.029       | 1            | 1.00         | 0.0000        | 0.000        | 0.000        | NA           |
| Na28  | 3             | 3            | 2.16         | 0.0872       | 3            | 2.23         | 0.0345       | 0.500        | 0.551        | 0.093        | 0.380         | 0.537        | 0.276       | 3            | 2.47         | −0.0565       | 0.700        | 0.595        | −0.176       |
| Mean  | 4.81          | 3.25         | 2.10         | −3.250       | 2.059        | 0.422        | (0.075)      | 0.420        | −0.006       | 0.417         | 0.406        | −0.001       | 2.94         | (0.392)      | 1.82         | −0.422        | 0.383        | 0.013        |
| (S.E.)| (0.857)       | (0.581)      | (0.351)      | (0.595)      | (0.253)      | (0.060)      | (0.110)      | (0.083)      | (0.060)      | (0.125)      | (0.170)      | (0.084)      | (0.057)      | (0.142)      |

Asterisks indicate significant differences from HWE equilibrium (Calculated from Chi\(^2\) tests) *P < 0.05, **P < 0.01; ***P < 0.001. Two loci, Na01 and Na14, are not shown since they were monomorphic in all sampled populations. Population abbreviations: CHA = Chanco; EMP = Empedrado; FED = Fundo el Desprecio; Voucher specimen numbers: CHA = CONC-CH 6002; EMP = No. CONC-CH 6003; FED = CONC-CH 6001.

N sample size, \(A_r\) total number of allele per loci across all populations, \(A\) number of alleles per locus, \(A_e\) effective number of alleles per locus, Null null alleles frequencies using Brookfield's estimator 1, according to [22]. \(H_0\) observed heterozygosity, \(H_e\) expected heterozygosity, \(F\) inbreeding coefficient, NA not defined.
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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human and animal participants This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent This research does not involve humans and therefore informed consents are not applicable.

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