Isolation and Characterization of Microsatellites For The Endangered Endemic Tree *Nothofagus Alessandrii* (Nothofagaceae)

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Short Communication

**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. Despite its conservation status, few molecular markers are available to study its population genetic, connectivity and to assist reproduction programs. 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 expected heterozygosity (H_E) of the Chanco population were 0.392 and 0.383. The mean values of H_O and H_E in the population of Empedrado (0.397 and 0.360, respectively) and Fundo el Desprecio (0.397 and 0.395, respectively) were similar. Linkage disequilibrium was found in a few pairs of loci suggesting that most of the markers can be considered as independent. Significant deviations from Hardy-Weinberg equilibrium ($P < 0.05$) were found in several 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 managements plans for the conservation on this endangered species.

Key Words: *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 (35-36°S). 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 narrow 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 over exploited 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, where most of its stands are secondary growth from stumps sprouts. In addition to those treats, the species shows low reproductive rates (i.e., low seed production and low seedling survival), which are likely to be the result of inbreeding due reduced population sizes and restricted gene flow caused by anthropogenic deforestation.

The aim of this study was to develop new microsatellite markers (simple sequence repeats SSRs) for *N. alessandrii*, using high-through-output 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 related to molecular ecology studies.

Material and Methods

DNA extraction and genome sequencing

Plant material for investigating the polymorphism in *N. alessandrii* was collected from three populations: (i) Los Ruiles National Reserve, sector “Chanco” (CHA), (ii) Los Ruiles National Reserve, sector “Empedrado” (EMP) and (iii) Fundo El Desprecio (FED) and (CHA: 35°50’S, 72°30’W; EMP: 35°37’S, 72°20’W, FED: 35°40’ S, 72°18’W). For each population young leafs from adult trees were collected and dried in silica gel before laboratory processing. Tissue from 58 individuals of *N. alessandrii* was collected for DNA extractions (n = number of individuals; CHA, n = 20; EMP: n = 20; FED, n = 18). On each population, a branch of one individuals was taken stored as a voucher in the Herbarium of the University of Concepción, Chillán, Chile (CHA: Voucher specimen No. CONC-CH 6002, EMP: Voucher specimen No. CONC-CH 6003, FED: Voucher specimen No. CONC-CH 6001). Additionally, to evaluate cross-species amplification in *N. pumilio* we used DNA extracted from ten individuals from Laguna Venus, Coyhaique (45°32’S, 72°00’W). Those DNA samples were part of a previous research [see reference 7]. In that study, the authors were unable to collect leaf tissue because branch and leafs were not accessible bellow 8-10 meters heights. Hence, DNA had to be extracted from cambium and phloem previously separated from bark, and no herbarium vouchers were 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 the Qubit® 3.0 (Invitrogen) fluorometric method. 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 QQD version v3.1 [8] was used to detect microsatellite motifs on reads higher than 80 pb. After this, BLASTn [9] we used to compare all sequences containing repeated motifs with at least 95% of similitude. This program identified 2,706 sequences containing short sequence repeats (SSR’s) 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 [10]. 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. MW387624–MW387641, Table 1).

PCR analyses and genotyping

For the amplification test, PCRs were performed in a final volume of 10 μ L containing: PCR, 10 μ M 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 30 s, specific annealing temperature (T_a) 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. 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 [11] was used to estimate the number of alleles per locus (A), the effective number of alleles per population (A_E), the observed heterozygosity (H_o), expected heterozygosity (H_E), inbreeding coefficient (F) and Hardy–Weinberg equilibrium (HWE) significance. Linkage disequilibrium was tested using GENEPOP version 4.2 [12]. Null allele frequencies were assessed in MICRO-CHECKER v2.2.3 [13] using Brookfield's estimator 1 [14]. The statistical significance was assessed using Bonferroni corrected P values.

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 amplified correctly (Table 1), and

sixteen of them were polymorphic (Table 2), including one tri-nucleotide and fifteen dinucleotide repeat markers. Considering all populations, a total of 77 alleles 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 2.03, 1.78, and 1.99 for CHA, EMP and FED, respectively (Table 2). For the entire dataset, the expected (H_E) and observed (H_O) 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 0.392 and 0.383, respectively. Similar values were found in populations EMP ($H_O = 0.397$, $H_E = 0.360$) and FED ($H_O = 0.397$, $H_E = 0.395$).

Significant deviations ($P < 0.05$) from Hardy–Weinberg equilibrium (HWE) were detected in several loci across populations (Table 2), possibly due to the small sampling size. Null alleles were detected in only three loci from CHA (*Na02*, *Na12*, *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.

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 km²) of its distributional range is under protection in the National System of Protected Areas (SNASPE) of Chile [15] and only two populations are considered within these protected areas: Los Ruiles National Reserve Empedrado (EMP) with 0.25 km² and Chanco (CHA) with 0.45 km² (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 [16] has addressed the genetic structure of the species but at a very limited extent (only seven fragments) and using allozyme's. Future studies should estimate the amounts of within and among population genetic diversity in the entire distributional range of this endangered species. The 16 markers developed in this study are the first specific

molecular marker developed for *N. alessandrii*. Those markers can be used to estimate the levels of genetic diversity of the species and to determine its genetic structure at different spatial scales. It is well known that this knowledge is pivotal to understand the evolutionary history of any species but also to develop management strategies aimed to preserve endangered species. For instance, those markers can be used to estimate the current levels gene flow within and among remnant fragments of by performing parentage/paternity analyses. In addition, they could be also used to evaluate whether the poor reproduction and survival currently found in this endangered species is related to anthropogenic habitat fragmentation. Together, these investigations should contribute to design appropriate conservation strategies for this endangered species.

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Compliance with ethical standards

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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