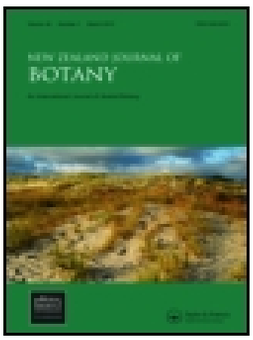

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SHORT COMMUNICATION

Characterisation and transferability of transcriptomic microsatellite markers for *Nothofagus* species

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ABSTRACT

Discriminant molecular markers are required for research on population genetics, as well as evolutionary studies involving identification of hybrids and parental species, or detection of the genome regions under selection. We provide a set of 27 transcriptomic microsatellite markers (SSRs) for South American *Nothofagus* species, derived from 73 *Nothofagus alpina (=N. nervosa)* annotated unigenes. Rates of cross-amplification ranged from 22% to 37%. Genetic characterisation of 22 transcriptomic SSRs for *N. alpina* and *N. obliqua* reveals low genetic variability, due to the general occurrence of one major allele at each locus, and high specificity, with few alleles shared between species (14%). At inter-species level 95% of loci were discriminant, with a total G''st over loci of 0.9, indicating that alleles were mostly fixed for all loci in both species. At intra-species level the number of markers with significant differentiation was 2.5 times higher for *N. obliqua* than for *N. alpina* populations. Moreover, transcriptomic SSRs showed higher performance compared with published anonymous microsatellites isolated from genome sequences without annotation. This set of transcriptomic microsatellites will be useful to the scientific community working on conservation and evolutionary aspects of *Nothofagus* species.

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Cross-amplification; discriminant markers; microsatellites; *Nothofagus alpina*; *Nothofagus obliqua*

Introduction

Nothofagus sensu lato (the only member of Nothofagaceae) is an ecologically and evolutionarily important genus in many temperate forests in the Southern Hemisphere. The genus is used as a model system for plant evolution (e.g. the controversy of vicariance vs. long distance dispersal), due to its significant role in historical biogeography and the numerous and diverse fossil records. Moreover, hybridisation is a common phenomenon among *Nothofagus* species; nevertheless, knowledge of its relevance in the adaptation and evolution of temperate forest tree species is still scarce.

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The generic name *Nothofagus* is currently under discussion. Heenan and Smissen (2013) presented a revision dividing the *Nothofagus* genus into four genera, whereas Hill et al. (2015) proposed to retain the previous genus *Nothofagus*. We agree with the arguments of Hill et al. (2015), therefore we have used the generic name *Nothofagus*. Microsatellites are one of the most popular molecular tools applied in population genetics and evolutionary biology studies (e.g. Varshney et al. 2005; Wang et al. 2009). For several applications the best microsatellite markers are those with a high effective number of alleles (e.g. fine-scale population structure, parentage and kinship analyses) (Abdul-Muneer 2014; Ashley 2010). In other cases, such as analysis of hybrid zones and adaptation studies, cross-amplification for multiple species and discriminant microsatellites are required (e.g. Vähä and Primmer 2006; Kane and Rieseberg 2007). Next-generation sequencing (NGS) technologies and bioinformatics tools have prompted the characterisation of molecular markers in non-model species in the last few years (e.g. Smissen et al. 2012; Huang et al. 2014). Using NGS, Torales et al. (2012) and El Mujtar et al. (2014) developed transcriptomic microsatellite markers (SSRs) for two South American tree species, *Nothofagus alpina* (Poepp. &Endl.) Oerst and *N. obliqua* (Mirb.) Oerst. However, these SSRs still require evaluation of the transferability and characterisation of genetic diversity among and within *Nothofagus* species.

Our aim in this work was to test cross-amplification of transcriptomic SSRs from *N. alpina* to other *Nothofagus* species, and their subsequent characterisation. We consider that focusing on putative functional regions related to differential traits within and among species may have higher potential for the development of discriminant markers than the use of randomly anonymous regions.

Materials and methods

We used 73 transcriptomic microsatellites (32 isotigs and 41 singletons) related to the abiotic stress category, which had been previously tested on six individuals of *Nothofagus alpina* (Torales et al. 2012).

DNA was extracted from leaf tissue of five *Nothofagus* species, according to the Marchelli et al. protocol (1998); *N. alpina* and *N. obliqua* belong to subgenus *Lophozonia*, whereas *N. antarctica* (G. Forst.) Oerst, *N. pumilio* (Poepp. & Endl.) Krasser and *N. dombeyi* (Mirb.) Oerst belong to subgenus *Nothofagus* (Manos 1997). Details of the geographic locations of sampled populations are shown in Table 1.

All *Nothofagus* species were used to test cross-amplification of the 73 transcriptomic markers, with *N. alpina* included as a positive reference. We used six individuals per species for the evaluation, which were mostly sampled from at least two populations (Table 1). Polymerase chain reaction (PCR) conditions and cycling programmes are reported in supplementary material as Document S1.

After PCR, amplicons were mixed with denaturing loading buffer, incubated for 5 min at 95 °C, and separated on a 6% polyacrylamide gel. Separated fragments were stained using the DNA silver staining procedure (Benbouza et al. 2006).

For each species we determined number of transferred transcriptomic SSRs, number of alleles and range of allele size per locus. Mean number of alleles over loci and number of shared alleles over loci and species were also calculated.

In addition, two populations from *N. alpina* (48 individuals) and two from *N. obliqua* (49 individuals) were genetically characterised using 22 polymorphic transcriptomic SSRs

Table 1. Geographic location of sampled populations.

Species	Population	Latitude	Longitude	Altitude (m.a.s.l)	Precipitation (mm/y)	Analysis
Nothofagus alpina	Hua Hum	40°07′55″	71°40′02″	940	2500	GC
	Tromen	39°36′00″	71°19′48″	1100	1750	GC
	Quilanlahue	40°08′21″	71°28′41″	930	1800	CA + GC
	Chidiak	40°08′57″	71°28′43″	650	1800	CA + GC
Nothofagus obliqua	Pilolil	39°30′05″	70°57′44″	836	700	GC
	Ñorquinco	39°09′11″	71°15′03″	1071	1500	GC
	Quilanlahue	40°08′21″	71°28′41″	930	1800	CA + GC
	Chidiak	40°08′57″	71°28′43″	650	1800	CA + GC
Nothofagus dombeyi	Quilanlahue	40°08′21″	71°28′41″	930	1800	CA
	Chidiak	40°08′57″	71°28′43″	650	1800	CA
Nothofagus antarctica	Tromen	39°36′00″	71°27′00″	850	>1000	CA
	Chalhuaco	41°14′00″	71°17′27″	1084	>1000	CA
Nothofagus pumilio	Tromen	39°44′47″	71°27′35″	1180	>1000	CA
	Chalhuaco	41°14′39″	71°17″09″	1122	>1000	CA
	Quilanlahue	40°07′59″	71°28′35″	1141	>1000	CA
	Lago Fontana	44°50′26″	71°37′58″	974	<900	CA
	Lago Guacho	43°48′53″	71°29′41″	1200	>1000	CA

GC, characterisation of genetic diversity; CA, cross-amplification; m.a.s.l, metres above sea level; mm/y, millimetres per year.

(Tables 1–2). The remaining SSRs were discarded due to genotyping problems (e.g. allele size > 300, both species sharing a fixed allele). PCR reactions were carried out using the same programmes and conditions as mentioned before. Amplified transcriptomic SSRs were pooled and genotyped, either as previously described, or with an ABI 3730 XL DNA Analyser (Applied Biosystems) by tagging forward primers with fluorescent dyes (FAM, HEX and NED). Raw data were analysed using software GeneMarker v1.97. Null alleles and genotyping errors were checked with Microchecker v2.2.3 (Van Oosterhout et al. 2004). Genetic diversity and differentiation estimates were determined with Genalex v6.5 (Peakall and Smouse 2012). Genetic differentiation was estimated by means of G"st (standardised Gst, based on expected heterozygosity) which takes into account the small number of populations and differences in sample size between populations.

Anonymous SSRs have been used to define genetic zones for management of *Nothofagus* genetic resources (Azpilicueta et al. 2013), allowing comparison between anonymous and transcriptomic SSRs. For this purpose, the genotype data of seven anonymous SSRs from the same populations as used for the genetic characterisation of 22 transcriptomic SSRs were required. Sample size was 85 for *N. alpina* (Hua Hum = 50 and Tromen = 35) and 66 for *N. obliqua* (Pilolil = 31 and Ñorquinco = 35). These data were re-analysed using the same software and estimators as mentioned before.

Results

Twenty-seven out of 73 transcriptomic SSRs were successfully transferred from *N. alpina* to four *Nothofagus* species (Table 2). Higher cross-amplification was observed for *N. obliqua* (37%) than for the remaining species (between 22% and 23%). Number of alleles and range of allele size revealed several loci showing variation among species. In particular, 81.5% of transcriptomic SSRs showed variation between *N. alpina* and *N. obliqua* in the level of polymorphism or allele size.

Number of alleles and number of effective alleles in *N. alpina* and *N. obliqua* populations revealed major alleles at high frequency for mostly all loci, determining low

Table 2. Cross-amplification of 27 SSRs derived from *Nothofagus alpina* annotated unigenes.

		Nothofagus alpina			*Nothofagus obliqua*			*Nothofagus dombeyi*			*Nothofagus antarctica*			*Nothofagus pumilio*		
ID name[a]	Locus	N	NA	RA	N	NA	RA	N	NA	RA	N	NA	RA	N	NA	RA
GR7D2IN01AUW3Ya	***2SN***	6	1	197	6	4	232–238	15	2	200–212	6	1	197	6	1	197
GR7D2IN01C09E8a	***11SN***	6	1	252	6	1	252	6	2	256–260	6	1	252	6	1	252
GR7D2IN01CGQUTa	***12SN***	6	2	230–236	6	2	230–236	6	1	225	6	1	220	6	1	225
GR7D2IN01D06Z8a	***13SN***	7	2	229–235	9	1	229	7	1	223	7	2	223–229	8	1	223
GR7D2IN01DG6LFa	***15SN***	6	1	166	6	1	173	6	1	166	6	1	166	6	1	166
GR7D2IN01E2MVEa	17SN	6	1	254	6	1	254	6	2	265–271	6	3	270–276	6	3	265–271
GR7D2IN01EMGE0a	***19SN***	7	3	155–161	9	7	155–167	7	5	149–167	7	7	159–175	8	6	153–169
GR7D2IN02FFJRFa	21SN	6	1	>330	6	1	>330	6	1	>330	6	2	>330	6	1	>330
GR7D2IN02FVL8Ua	***23SN***	7	3	118–124	9	2	115–118	7	2	112–115	7	3	109–115	8	1	115
isotig00230a	***IN0230a***	7	2	111–117	9	2	97–99	7	1	99	7	1	99	8	1	99
isotig00580a	***IN0580a***	6	1	165	6	1	169	6	1	162	6	1	164	6	2	166–170
isotig01207a	***IN1207a***	6	1	275	6	2	260–266	6	2	254–260	6	1	275	6	2	255–261
isotig00192a	IN0192a	6	1	139	12	2	141–143	6	1	139	6	1	139	12	2	139–143
isotig00600a	IN0600a	12	1	>330	12	4	>330	6	4	>330	6	4	>330	12	3	>330
GR7D2IN02H9XYAa	31SN	6	1	202	6	1	202	6	3	195–203	6	3	202–218	6		
isotig00842a	***IN0842a***	12	2	259–262	6	2	259–265	6	2	270–279	6			6	2	270–279
isotig00903a	***IN0903a***	6	1	274	12	3	271–280	6	2	259–274	6			12	2	274–283
isotig00193a	***IN0193a***	6	1	148	12	2	142–145	6			6	1	130	6		
GR7D2IN01BK031a	***7SN***	6	1	230	6	2	228–231	6			6			6		
GR7D2IN01BK031b	***8SN***	7	2	238–240	9	1	230	7			7			8		
GR7D2IN02HOKOIa	***32SN***	7	2	112–121	9	1	121	7			7			8		
GR7D2IN02HWWWTa	***35SN***	6	1	251	6	1	263	6			6			6		
GR7D2IN02IGGX9a	***38SN***	6	1	160	6	3	154–160	6			6			6		
GR7D2IN02IZPUXb	***41SN***	6	1	101	6	2	98–107	6			6			6		
isotig00597a	***IN0597a***	7	2	223–227	9	3	219–227	7			7			8		
isotig00192b	***IN0192b***	7	1	148	9	1	145	7			7			8		
isotig01459a	***IN1459a***	12	2	130–133	12	2	156–159	6			6			6		
mean over loci			1.44			2.04			1.94			2.06			1.88	

N, sample size; NA, number of alleles; RA, range of allele size (in base pairs). Loci selected for characterisation of genetic variability in *N. alpina* and *N. obliqua* are indicated in italics and bold type.
[a]ID SSRs in *N. alpina* from Torales et al. (2012).

genetic diversity for these species. This pattern was also reinforced by low levels of observed and unbiased expected heterozygosity (Table 3). Considering both species the total number of alleles was 120, with only 14% being shared between species. Mean number of alleles and private alleles over loci were 2.59 and 2.45 for *N. obliqua* and 2.00 and 1.36 for N. *alpina*, respectively; indicating a clear difference in the distribution of alleles between species. High genetic differentiation was shown by 95% of loci. Total G"st was 0.939 (p-value < 0.001), with a minimum G"st value of 0.322. No correlation was observed between estimates of differentiation and genetic diversity, as high differentiation was observed for polymorphic and monomorphic loci (Figure 1).

Evidence of inbreeding was not detected (Fis < 0.02; Table 3). Null alleles (frequencies >10%) were detected at four loci in *N. alpina*, IN0580a in Tromen, 15SN in Hua Hum, IN1459a and IN0597a in both populations, and only at locus 12SN for *N. obliqua* in both populations. Although corrections for null alleles were not implemented we considered that patterns of differentiation at inter- and intra-species levels were generally not affected by their occurrence, because: 1. five loci with null alleles did not share alleles between species; 2. differentiation was not observed in *N. alpina* for the four loci with null alleles; and 3. allele range of the locus with null alleles differed between *N. obliqua* populations.

Mean number of alleles and private alleles over loci obtained here were 5.29 and 2.00 for *N. obliqua* and 5.71 and 2.43 for *N. alpina*, respectively, indicating a partial overlap of allele distributions between species. In fact, the percentage of alleles shared for both species was twice as high for anonymous SSRs as for transcriptomic SSRs (Table 4). Both markers were tested for the same two populations of *N. alpina* and *N. obliqua*. This pattern was reinforced by differentiation estimate, indicating that efficiency of development of discriminant SSRs at inter-species level (mostly fixed markers between species) was 2.5 times higher for transcriptomic SSRs than for anonymous markers (Table 4).

To sum up, these results showed higher discriminant capacity in markers derived from unigenes of the abiotic stress category than from anonymous markers.

Discussion

The global rate of cross-species amplification in our study was 37% (27 out of 73 tested SSRs). Smissen et al. (2012) reported transferability of 44 anonymous SSRs from *Nothofagus solandri* to two other species of subgenus *Fuscospora*, obtaining 16% polymorphic transferred SSRs. The high level of transferability of microsatellites from transcriptome sequences is attributable to the nature of their primers, which are developed from gene sequences; however, it also depends on the genetic distance between species (Dufresnes et al. 2014). In this sense, marker transferability in our study reflects divergence time (Sauquet et al. 2012), since cross-amplification was higher among *Lophozonia* species than between species of subgenus *Lophozonia* and subgenus *Nothofagus*.

The polymorphism of transferred transcriptomic SSRs was globally low in the *Nothofagus* species analysed here (mean number over loci and species <2.6; Tables 2–3). A similar result was observed for mean number of alleles (<4.0, El Mujtar et al. 2014) and mean number of effective alleles (<2.1, El Mujtar et al. 2014; <4.0, Soliani et al. 2015) of anonymous markers. This should not be assumed to be a direct consequence of using transcriptomic markers, although selection on coding regions could also influence diversity

Table 3. Genetic diversity estimates of 22 analysed transcriptomic SSRs for *Nothofagus alpina* and *Nothofagus obliqua*.

	Nothofagus alpina										Nothofagus obliqua									
	Hua Hum					Tromen					Pililil					Ñorquinco				
Locus name	N	Na	Ne	Ho	He	N	Na	Ne	Ho	He	N	Na	Ne	Ho	He	N	Na	Ne	Ho	He
IN0580a	22	1	1.00	0.00	0.00	24	***2***	***1.18***	***0.00***	***0.16***	26	1	1.00	0.00	0.00	23	2	1.09	0.09	0.09
IN0842a	19	2	1.50	0.32	0.34	23	2	1.87	0.39	0.48	25	4	2.18	0.76	0.55	21	4	1.56	0.33	0.37
IN1459a	23	***3***	***2.23***	***0.00***	***0.56***	*19*	***3***	***2.64***	***0.00***	***0.64***	23	1	1.00	0.00	0.00	19	1	1.00	0.00	0.00
IN1207a	24	2	1.04	0.04	0.04	24	1	1.00	0.00	0.00	24	2	1.28	0.25	0.22	23	2	1.14	0.13	0.12
38SN	24	1	1.00	0.00	0.00	24	1	1.00	0.00	0.00	24	1	1.00	0.00	0.00	22	1	1.00	0.00	0.00
7SN	24	1	1.00	0.00	0.00	24	1	1.00	0.00	0.00	23	1	1.00	0.00	0.00	22	1	1.00	0.00	0.00
2SN	24	1	1.00	0.00	0.00	15	1	1.00	0.00	0.00	23	12	7.50	0.83	0.89	19	11	6.56	0.84	0.87
IN0903a	24	1	1.00	0.00	0.00	23	1	1.00	0.00	0.00	23	2	1.14	0.13	0.12	14	3	1.24	0.07	0.20
41SN	24	1	1.00	0.00	0.00	24	1	1.00	0.00	0.00	24	2	1.38	0.17	0.28	23	2	1.46	0.39	0.32
15SN	22	***4***	***1.82***	***0.27***	***0.46***	24	5	2.37	0.71	0.59	23	2	1.09	0.09	0.09	23	1	1.00	0.00	0.00
11SN	24	1	1.00	0.00	0.00	24	1	1.00	0.00	0.00	20	3	1.29	0.25	0.23	23	2	1.52	0.35	0.35
IN0193a	24	1	1.00	0.00	0.00	24	1	1.00	0.00	0.00	26	1	1.00	0.00	0.00	22	1	1.00	0.00	0.00
35SN	24	1	1.00	0.00	0.00	24	1	1.00	0.00	0.00	26	1	1.00	0.00	0.00	23	1	1.00	0.00	0.00
12SN	23	1	1.00	0.00	0.00	23	1	1.00	0.00	0.00	23	***6***	***3.88***	***0.17***	***0.76***	23	***4***	***2.56***	***0.39***	***0.62***
13SN	22	4	2.37	0.55	0.59	24	3	1.98	0.42	0.50	24	1	1.00	0.00	0.00	23	1	1.00	0.00	0.00
19SN	21	4	2.31	0.52	0.58	23	6	2.20	0.52	0.56	25	8	4.24	0.72	0.78	20	5	4.06	0.60	0.77
23SN	23	3	2.16	0.57	0.55	24	3	1.90	0.50	0.48	24	2	2.00	0.54	0.51	19	2	1.95	0.32	0.50
IN0597a	20	***3***	***2.46***	***0.40***	***0.61***	23	***3***	***2.07***	***0.30***	***0.53***	16	3	1.14	0.06	0.12	19	9	3.17	0.53	0.70
32SN	23	2	1.29	0.26	0.23	22	2	1.94	0.55	0.49	24	1	1.00	0.00	0.00	19	1	1.00	0.00	0.00
IN0192b	23	2	1.14	0.13	0.12	24	1	1.00	0.00	0.00	23	1	1.00	0.00	0.00	23	1	1.00	0.00	0.00
IN0230a	22	3	1.26	0.23	0.21	22	2	1.37	0.32	0.27	25	1	1.00	0.00	0.00	21	1	1.00	0.00	0.00
8SN	23	2	1.24	0.22	0.20	23	2	1.78	0.57	0.45	24	1	1.00	0.00	0.00	22	1	1.00	0.00	0.00
Mean																				
over loci		2.00	1.40	0.16	0.20		2.00	1.47	0.19	0.23		2.59	1.73	0.18	0.21		2.59	1.70	0.18	0.22
over loci and pops		2.00	1.43	0.18	0.22							2.59	1.71	0.18	0.21					
Fis	0.02 (0.02)					0.01 (0.01)					0.01 (0.01)					0.02 (0.02)				

N, sample size; NA, number of alleles; Ne, number of effective alleles; Ho and He, observed and unbiased expected heterozygosity; Fis, estimated inbreeding coefficient for each population (number in parentheses indicates standard error). Loci with null allele are indicated in italics and bold type.

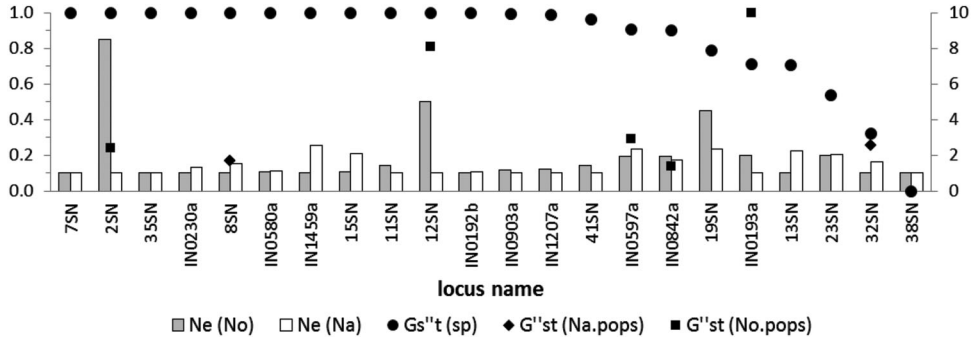

Figure 1. Estimates of differentiation and genetic diversity of 22 transcriptomic SSRs. G''st: Hedrick's standardised Gst, estimate of genetic differentiation (on principal axis). G''st (sp): inter-species divergence. G''st (Na.pops): differentiation between populations of *N. alpina* (Na). G''st (No.pops): differentiation between populations of *N. obliqua* (No). Ne: Number of effective alleles (on secondary axis). Only G''st with significant *p*-values (<0.05) are presented.

Table 4. Comparison between markers: transcriptomic SSRs and anonymous SSRs.

Parameter[a]	Transcriptomic SSRs (22)	Anonymous SSRs (7)
Shared alleles	14.2	29.9
G''st inter-sp > 0.5	90.5	71.4
G''st inter-sp > 0.9	71.4	28.6
G''st intra-Na	9.5	42.9
G''st intra-No	23.8	0.0

[a]Values of all parameters are indicated as a percentage of the total number of loci which are indicated between parentheses. For the 'shared alleles', parameter percentage was calculated using total number of alleles over loci. G''st inter-sp indicates genetic differentiation estimates at inter-species level higher than 0.5 or 0.9 respectively. G''st intra-Na or intra-No indicates genetic differentiation estimates at intra-species level; that is, considering divergence between the populations of each species. Only loci G''st with significant *p*-value (< 0.05) were considered.

patterns. The low genetic variability, due to the occurrence of mostly one major allele at each locus, was reinforced by fewer alleles shared between species for transcriptomic SSRs (14%) than for anonymous SSRs (30%).

Nothofagus alpina and *N. obliqua* are tree species of southern South American temperate forests which occur along a wide rainfall gradient. In Argentina the species' range follows west-east oriented water-sheds and lies along altitudinal gradients, occurring in sympatry over approximately 18,000 ha, either as mixed or pure forests (Sabatier et al. 2011). Despite their sympatric regional distribution, the species present ecological and physiological differences (Varela et al. 2010; Azpilicueta et al. 2013; Arana et al. 2016), suggesting adaptive variation. The discriminant transcriptomic SSRs we have characterised will therefore be useful in ecological and evolutionary genetic studies of these species (e.g. identification and classification of hybrids, evaluation of patterns of introgression for hybrid zones and studies of local adaptation).

Finally, we consider it important to highlight that level of polymorphism has traditionally been used as a criterion for selecting candidate SSRs for subsequent characterisation (Queirós et al. 2015; Vukosavljev et al. 2015). Targeting high levels of polymorphism may

not be the best option when searching for markers that differ between species, since high polymorphism is not expected for the genome regions under selection. In our study, 63% (17 out of 27) of transferred transcriptomic SSRs were monomorphic for the source species (*N. alpina*), whereas 81.5% (22 out of 27) showed differences in the range of allele size or polymorphism when transferred to four related species.

We tested the performance of our strategy on transcriptomic SSRs selected from abiotic stress unigenes; however, this criterion could be changed according to the goals of specific studies.

Conclusion

We provide a set of microsatellites for *Nothofagus* species which will be useful for conservation and evolutionary studies, and demonstrate that discriminant markers can be developed efficiently by mining functional annotation of transcriptome sequences.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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