

RESEARCH ARTICLE

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# Transcriptome survey of Patagonian southern beech *Nothofagus nervosa* (= *N. Alpina*): assembly, annotation and molecular marker discovery

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## Abstract

**Background:** *Nothofagus nervosa* is one of the most emblematic native tree species of Patagonian temperate forests. Here, the shotgun RNA-sequencing (RNA-Seq) of the transcriptome of *N. nervosa*, including *de novo* assembly, functional annotation, and *in silico* discovery of potential molecular markers to support population and associations genetic studies, are described.

**Results:** Pyrosequencing of a young leaf cDNA library generated a total of 111,814 high quality reads, with an average length of 447 bp. *De novo* assembly using Newbler resulted into 3,005 tentative isotigs (including alternative transcripts). The non-assembled sequences (singletons) were clustered with CD-HIT-454 to identify natural and artificial duplicates from pyrosequencing reads, leading to 21,881 unique singletons. 15,497 out of 24,886 non-redundant sequences or unigenes, were successfully annotated against a plant protein database. A substantial number of simple sequence repeat markers (SSRs) were discovered in the assembled and annotated sequences. More than 40% of the SSR sequences were inside ORF sequences. To confirm the validity of these predicted markers, a subset of 73 SSRs selected through functional annotation evidences were successfully amplified from six seedlings DNA samples, being 14 polymorphic.

**Conclusions:** This paper is the first report that shows a highly precise representation of the mRNAs diversity present in young leaves of a native South American tree, *N. nervosa*, as well as its *in silico* deduced putative functionality. The reported *Nothofagus* transcriptome sequences represent a unique resource for genetic studies and provide a tool to discover genes of interest and genetic markers that will greatly aid questions involving evolution, ecology, and conservation using genetic and genomic approaches in the genus.

**Keywords:** Nothofagaceae, Forest genomics, Pyrosequencing, *de novo* transcriptome assembly, SSRs, Functional annotation

## Background

The Nothofagaceae family contains only the genus *Nothofagus*, and comprises 36 recognized species, 26 of which occur in Australia and the remaining 10 in South

America [1]. *Nothofagus* in Argentina is represented by only six endemic species, distributed on the foothills of the Andes and surrounding valleys, beginning with its appearance at 36° in the province of Neuquen, and extending to 55°S, in the province of Tierra del Fuego [2].

Among these species, *N. obliqua*, *N. nervosa* and *N. pumilio*, occupy a relatively precise range within an altitudinal gradient spanning from 600 m over the sea level up to 1800 m. Along this gradient each species withstand different environmental conditions, especially extremely

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cold temperatures at the higher altitudes. Individual trees living in this environmental gradient, exhibit adaptive features for adverse conditions such as drought and extreme temperatures, traits that may prove value for adapting to future climate changes in the context of global climate change.

*N. nervosa* (Phil.) Dim.et Mil [3] (= *N. alpina* (Poepp. & Endl.) Oerst) commonly known as “rauli”, is one of the most important species of Patagonian Temperate Forests due to its wood quality and its relatively fast growth [4]. In Argentina it covers a reduced area, only 79,636 hectares in a narrow fringe of about 120 km in length and about 40 km in maximum width [5,6]. This deciduous species suffered a great overexploitation in the past due to its high wood quality, making necessary to implement conservation policies and management programs [7].

The distribution of adaptive genetic variation is an importance issue in forest species, both native and domesticated, serving as a basis for natural resource management and conservation genetics [8]. The characterization of genetic diversity is also important in order to determine its relation with phenotypic variation [9]. Massive sequencing techniques are among the new strategies used in functional genomics for gene discovery and molecular markers development in non-model organisms or in those species whose genomes have not been completely sequenced. It provides a fast and effective way to get new genetic information of an organism and allows a rapid access to a collection of expressed sequences (transcriptome).

To date, model forest tree species belonging to *Eucalyptus* genus [10-12], *Pinus*, *Picea* and *Populus* [13-17] have comprehensive transcriptome information.

The Fagaceae family (represented by the genus *Quercus*, *Castanea* and *Fagus*) also holds a large number of sequenced transcripts with approximately 2.5 millions of ESTs deposited in databases (Fagaceae Genomics Web: <http://www.fagaceae.org/>). At present, new sequencing

technologies offer the possibility to obtain gene catalogs for non-model organism which is an opportunity for forest tree transcriptome characterization, discovery of alternative metabolic strategies and functional molecular markers [9].

One of the advantages of transcriptome pyrosequencing is in terms of sequence reliability. Each region of the cDNA is read several times in both strands compared to one sequence/one strand reading of conventional ESTs.

In this study we characterized leaf *N. nervosa* transcriptome by pyrosequencing and analyzed the resulting sequence data. Moreover, the functional annotation of the unigenes, allowed us to have a global but throughout picture of leaf functional gene expression, as well as to deduce the metabolic pathway represented in this dataset.

This information will significantly contribute to the development of *Nothofagus* functional genomics, genetics and population-based genome studies. In addition, the rather limited set of molecular markers available until now: 14 microsatellites isolated from *N. cunninghamii* [18], 11 developed in six species of South American *Nothofagus* [19], five in *N. nervosa* [20], and nine microsatellite loci from *N. pumilio* [21], will be substantially increased with thousands of new markers, both from neutral and functional sequences. The quality of the sequence information here reported was confirmed by the successful PCR amplification of molecular markers using oligonucleotide primers designed with the deduced sequences.

## Results and discussion

### Transcriptome sequencing and assembly

Pyrosequencing of cDNA on a 454 GS FLX Titanium (Roche) generated a total of 146,267 raw reads, with an average length of 408 bp. After filtering for adaptors, primer and low-quality sequences, 5,588 reads were removed resulting in 140,679 high quality reads corresponding to 96% of the first raw sequences, representing

**Table 1 *N. nervosa* transcriptome annotation summary**

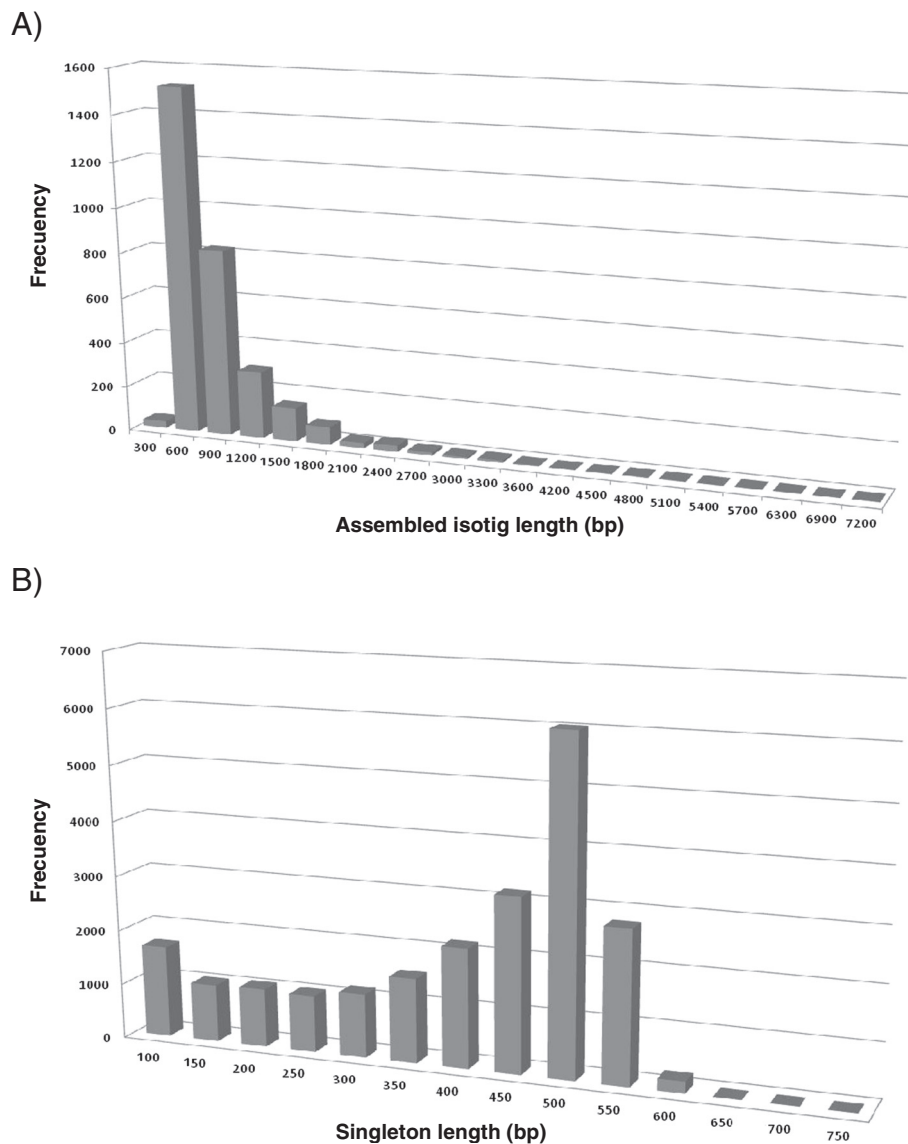
	Number of sequences		
	Isotigs (3,005)	Singletons (21,881)	Combined set (24,886)
<b>Viridiplantae-NR</b>			
Sequences with positive BLAST matches	2,762 (92%)	12,735 (58%)	15,497 (62%)
Sequences annotated with Gene Ontology (GO) terms	2,238 (74%)	9,596 (44%)	11,834 (47%)
Sequences without detectable BLAST matches	243 (8%)	9,146 (42%)	9,389 (38%)
Sequences assigned to know Enzyme Commission category	931 (31%)	1,424 (6%)	2,355 (9%)
<b>Fagaceae</b>			
Sequences with positive BLAST matches	2,923 (97%)	17,515 (80%)	20,438 (82%)
Sequences without detectable BLAST matches	82 (3%)	4,365 (20%)	4,447 (18%)
Sequences annotated with Gene Ontology (GO) terms (“novel genes”)	12 (0.4%)	490 (2%)	502 (2%)

Numbers and percentages of 454 sequences in the assembled isotigs, singletons and unigenes with significant matches against NCBI NR proteins Viridiplantae filtered database and Fagaceae unigenes.

approximately 60 Mbp. Raw data (>200 bp) were deposited in NCBI Sequence Read Archive (SRA) under the accession number SRA049632.2.

By using Newbler Software v. 2.5 (Roche, IN, USA); a total of 111,814 sequences were *de novo* assembled into 3,394 contiguous sequences (contigs). Overlapping contigs were assembled into 3,005 isotigs (equivalent to unique RNA transcripts). In addition, isotigs originating from the same contig-graph were grouped into 2,722 isogroups (equivalent to genomic locus) by Newbler, potentially reflecting multiple splice variants. About 28,861 reads not assembled into isotigs were clustered using CD-HIT-454 algorithm to eliminate artificial duplicates leaving 21,881 singletons, summing up a total of 24,886

non-redundant sequences or unigenes (Table 1). All unigene sequences (isotigs and singletons >200 bp) were deposited to the Transcriptome Shotgun Assembly (TSA) database, accession numbers JT763459-JT784547. Isotig length ranged from 66 bp to 7,093 bp, with an overall average length of  $765 \pm 537$  bp (Figure 1A). More than 83% of the isotigs were 66 to 1,000 bp long and 50% of the assembled bases were incorporated into isotigs greater than 589 bp. The average length of *N. nervosa* isotigs (765 bp) was larger than those assembled in other non model organisms (e.g. 197 bp [22], 440 bp [23], 500 bp [24]; 535 bp, [25]), and similar to the average isotig length described in *Bituminaria bituminosa* (707 bp [26]).



**Figure 1** Frequency distribution of isotigs (A) and singletons (B) sequences length. The histograms represent the number of isotig and singletons sequences in relation to its length.

The coverage depth for isotigs ranged from 2 to 19, with an average of 9 contigs assembled into each isotig, which is larger than the averages obtained in other 454 transcriptome analyses (mean = 2.1, [24,25]).

The length distribution of the 21,881 singletons ranged from 50 to 711 bp with an overall average length of 369.6 bp (Figure 1B). The length of 86% of the singletons was shorter than 500 bp.

### Functional annotation

All unique sequences were subjected to BLASTX similarity search against the NR protein database (National Center for Biotechnology Information, NCBI), with a Viridiplantae filter, to assign a putative function [27].

Under an E-value threshold of  $<10^{-10}$ , a total of 2,762 isotigs (92% of total isotigs) and 12,735 singletons sequences (58% of total singletons) had significant BLASTX matches (Table 1). The frequency of annotated isotigs was significantly higher than the values previously reported for *de novo* transcriptome assemblies of eukaryotes that range from 20 to 40% [22-25].

In total, 15,497 unique sequences had at least one hit, while the remaining sequences 9,389 (38%) exhibited less significant matches (e-value  $>10^{-10}$ ) but still informative for identifying putative biological functions in future studies in this species. We also performed a BLASTX against the NCBI - NR protein database to retrieve sequences that did not show BLAST hits against Viridiplantae NCBI, which summed up some few new hits (81), but not adding any other valuable annotations.

The majority of matched sequences exhibited high similarity to *Vitis vinifera* (41%), and *Populus trichocarpa* (38%) sequences. The top-hit species distribution of BLAST matches is shown in Figure 2.

Annotation and mapping routines were run with BLAST2GO platform [28]. Sequences with a positive BLAST match were annotated using Gene Ontology terms (GO) and Enzyme Commission categories (i.e. EC numbers). Thus, GO terms were assigned to 2,238 isotigs (74%) and 9,596 singletons (44%) totalizing 11,834 GO terms (Table 1).

Of the 11,834 GO annotated isotigs and singletons sequences, most were assigned to "Biological Processes" (7,926 terms), "Molecular functions" (8,229 terms) and "Cellular Components" (9,206 terms), (Figure 3).

BLAST2GO analysis at process level 2, showed that among 21 different biological processes most of the transcripts belonged either to "Metabolic Processes" (5,823), to "Cellular Processes" (5,090) and to "Response to Stimuli" (1,493), of which 756 were putative stress-response genes (Figure 3A).

Likewise, the molecular function category subdivided annotated sequences into binding (6,985), catalytic

activity (5,658) and transporters (689) as the most represented (Figure 3B).

A detailed BLAST2GO analysis (level 2) at the cellular component category, sorted all transcripts from *N. nervosa* into 5 groups being the most representative: cell (7,304), organelle (4,822) and macromolecular complex component (1,136) (Figure 3C).

In order to more precisely compare the similarity of *N. nervosa* genes with those of the Fagaceae family (from Fagaceae Genomics Web [http://www.fagaceae.org/]), *N. nervosa* unigenes were subjected to BLAT (dnax) search against 2,407,823 contigs and singletons from American Beech (*Fagus grandiflora*), American Chestnut (*Castanea dentate*), Chinese Chestnut (*Castanea mollissima*) and oak species (*Quercus rubra* and *Q. alba*). Eighty-two percent of the *N. nervosa* expressed sequences exhibited high similarity to Fagaceae genes. A total of 4,447 (18%) sequences did not show matches against Fagaceae sequences, from which there were 82 isotigs and 4,365 singletons. Among them, 12 isotigs and 490 singletons had distinctive GO annotation, which could be considered as novel genes for this large group of tree species (Table 1). Most interestingly, from these transcripts 21 were found to be potentially new genes for stress response (data not shown).

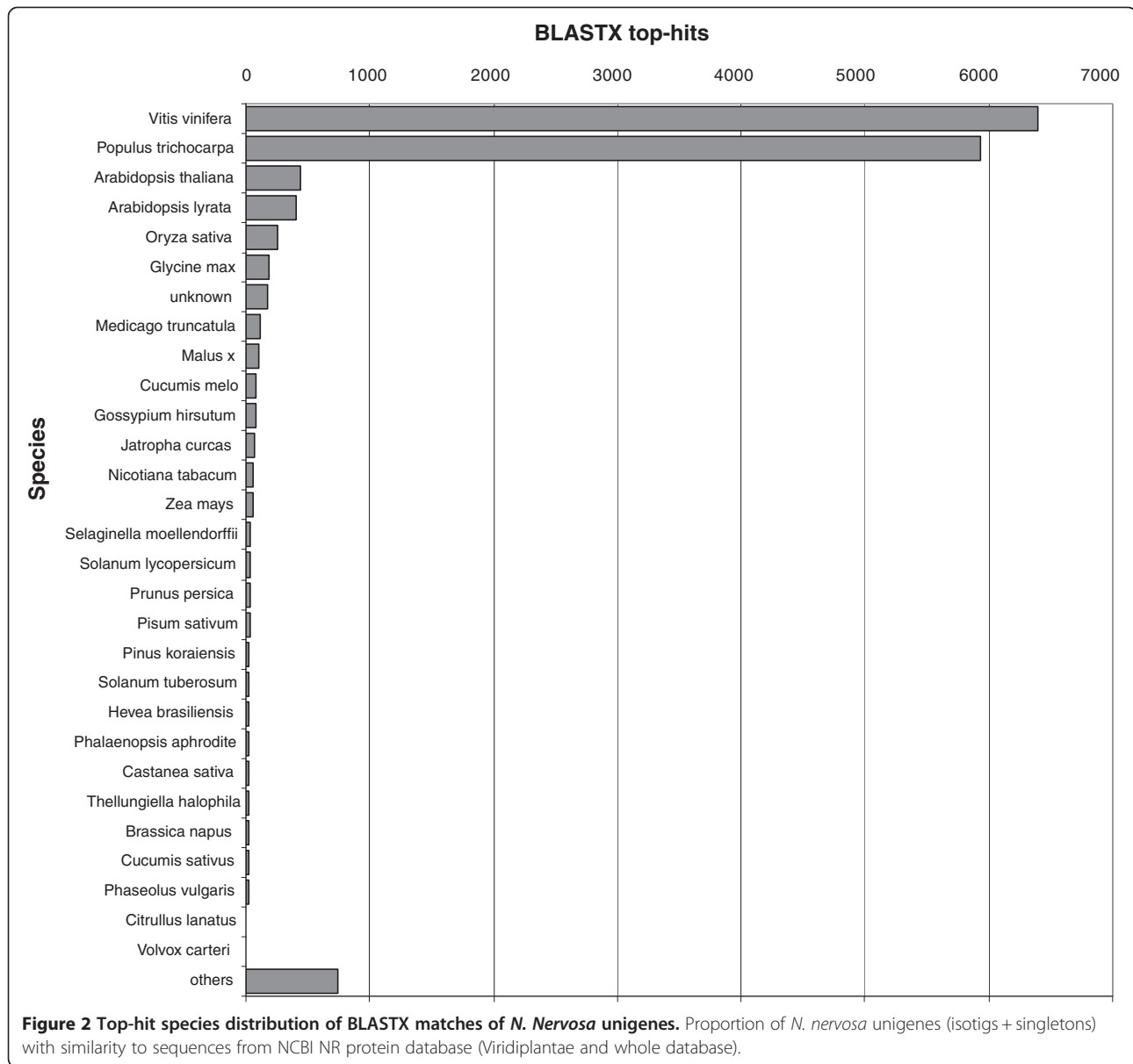
Of the 11,834 sequences annotated with GO terms, 2,355 were assigned with EC numbers (931 isotigs and 1,424 singletons) (Table 1).

The most represented enzymes in all sequences are shown in Figure 4: transferase activity (37%), hydrolase activity (35%) and oxidoreductase activity (13%) were the most abundant.

To further enhance the annotation of *N. nervosa* transcriptome dataset, the 11,834 genes with GO terms were mapped to KEGG using KEGG automatic annotation server (KAAS) [29]. The identified 58 metabolic pathways include: purine metabolism (411), thiamine metabolism (405), T cell receptor signalling pathway (115), biosynthesis of secondary metabolites (58), and microbial metabolism in diverse environments (37) (see Additional file 1).

We detected as much as 861 chloroplast (cp) sequences (150 in isotigs and 711 in singletons), corresponding to a quite high rate (7%), but this value was within the 2 to 10% found in cDNA libraries from all tissue types, as reported in a study conducted in oak [30].

The number of annotated isotigs in this study was comparatively larger than that obtained in other similar studies [22-25]. These results could be associated with the high quality and small number of assembled isotigs, which potentially corresponds to highly expressed genes. Also the use of specific plant protein sequences and close related Fagaceae database possibly increased the



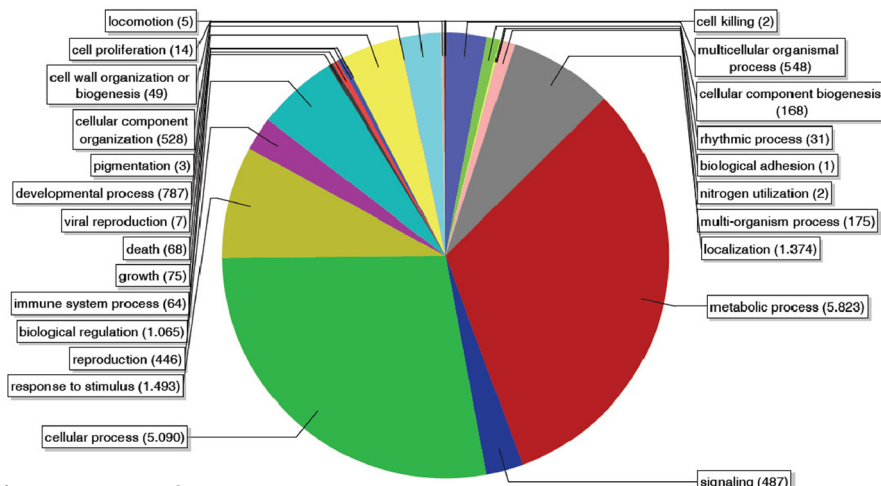
BLAST hits. The first assumption comprises technical issues such as a high percentage of isotigs that was greater than ~600 bp length and with good coverage depth. Moreover, the small number of isotigs would be detecting the most represented and known expressed genes, as it was also shown in the analyses of *B. bituminosa* leaf transcriptome (89.1% annotated contigs) [26]. Proportions of best hits in major GO category were generally similar to those found in this species, for example, binding 48% and catalytic activity 37% in the *N. nervosa* transcriptome survey versus 37% and 37% respectively for the same categories in *B. bituminosa*.

The second statement relies on the annotation approach based on the search against the Viridiplantae

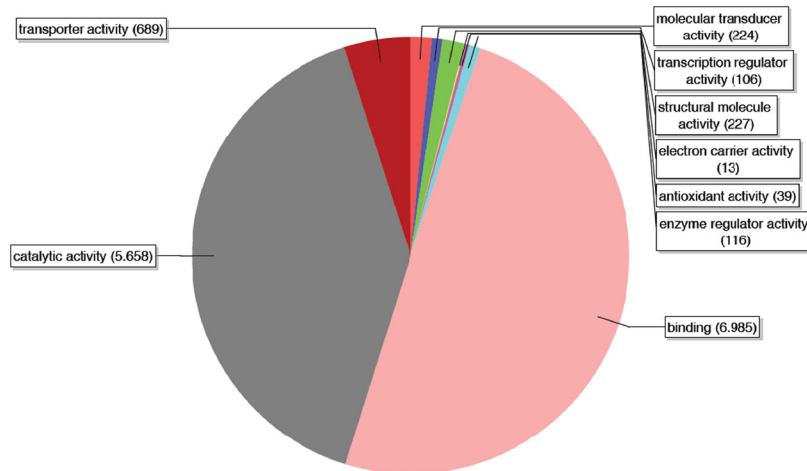
protein database. This strategy allows to more likely finding BLAST hits above the cut off value. In addition, a higher percentage of reliable annotated isotigs was found when the searched was carried out against the Fagaceae protein sequence dataset (Table 1). The favorable effect of using specific databases for annotation was also reported for other authors [31-33].

Besides, the lower percentage of singletons that were annotated was likely due to the high frequency of short length sequences, also reported in recent studies [24,34]. Fifty percent of non-annotated singletons were shorter than 370 bp (data not shown), whereas the 50% in annotated singletons were longer than 454 bp. Similar results were obtained in *Pinus contorta* where only 5% of

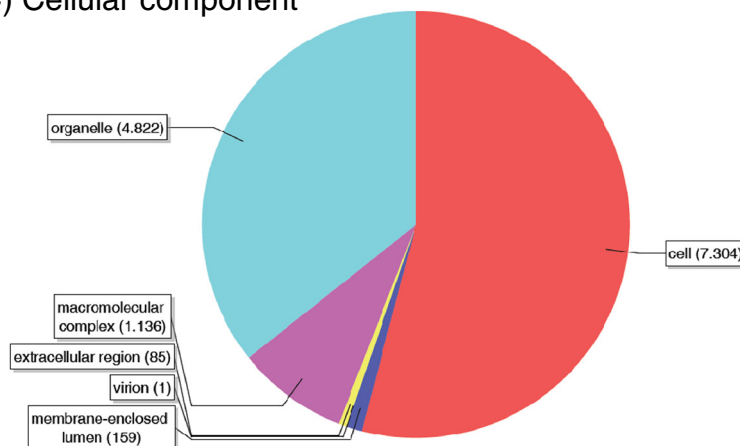
### A) Biological process



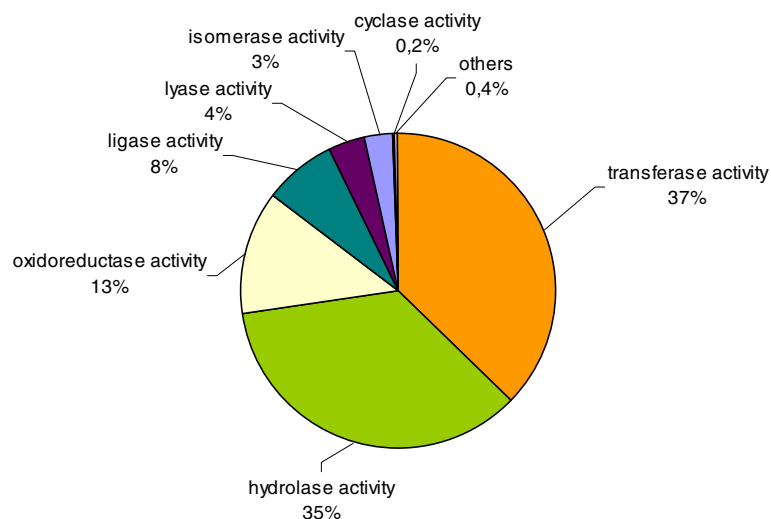
### B) Molecular function



### C) Cellular component



**Figure 3 Gene Ontology (GO) assignment in level 2 of 11,834 *N. nervosa* unigenes.** The total numbers of unigenes annotated for each main category are 7,926 for "Biological Process" (A), 8,229 for "Molecular Function" (B), and 9,206 for "Cellular Component" (C).



**Figure 4** Catalytic activity distribution in annotated *N. nervosa* unigenes.

contigs and singletons had BLAST matches when the length of the sequences was less than 250 bp [24]. Nonetheless, many singletons were good quality reads and matched to proteins in BLAST searches representing together with the isotigs, a great source of information.

Summarizing, the frequency of annotated isotigs and singletons was significantly higher than previously reported for new generation sequencing *de novo* transcriptome assemblies of trees like *Pinus contorta* [24], or two oaks species, *Quercus petraea* and *Q. robur* [30], even though the high stringency of BLASTX analysis.

If we assume that the average number of genes encoded in a plant nuclear genome is about 30 thousands (as estimated from seven completely sequenced genomes) [34], our annotated dataset likely represents a half of the *N. nervosa* genes catalogue.

In order to test the presence of expressed repetitive sequences, BLASTN (e-value cut off  $\leq 10e^{-50}$ ) searches were performed against all Viridiplantae Repbase (reference database of eukaryotic repetitive DNA). A total of 374 repetitive DNA sequences were found (57 in isotigs and 317 in singletons). From all the rRNA sequences, 255 corresponded to small subunit rRNA (SSUrRNA), 102 to large subunit rRNA (LSUrRNA) and 17 to transposable elements. Similar numbers of retrotransposon were observed in other plant species (e.g. 15 in *Populus tremula* and *Pinus pinaster*) [24]. However, in *Fagopyrum esculentum* and *Pinus contorta* much more transcribed retrotransposable elements were found in the different tissues sampled [24,34].

#### **In silico mining of single sequence repeats (SSRs)**

Using the SSR webserver from the Genome Database for Rosaceae (GDR), we identified and characterized several

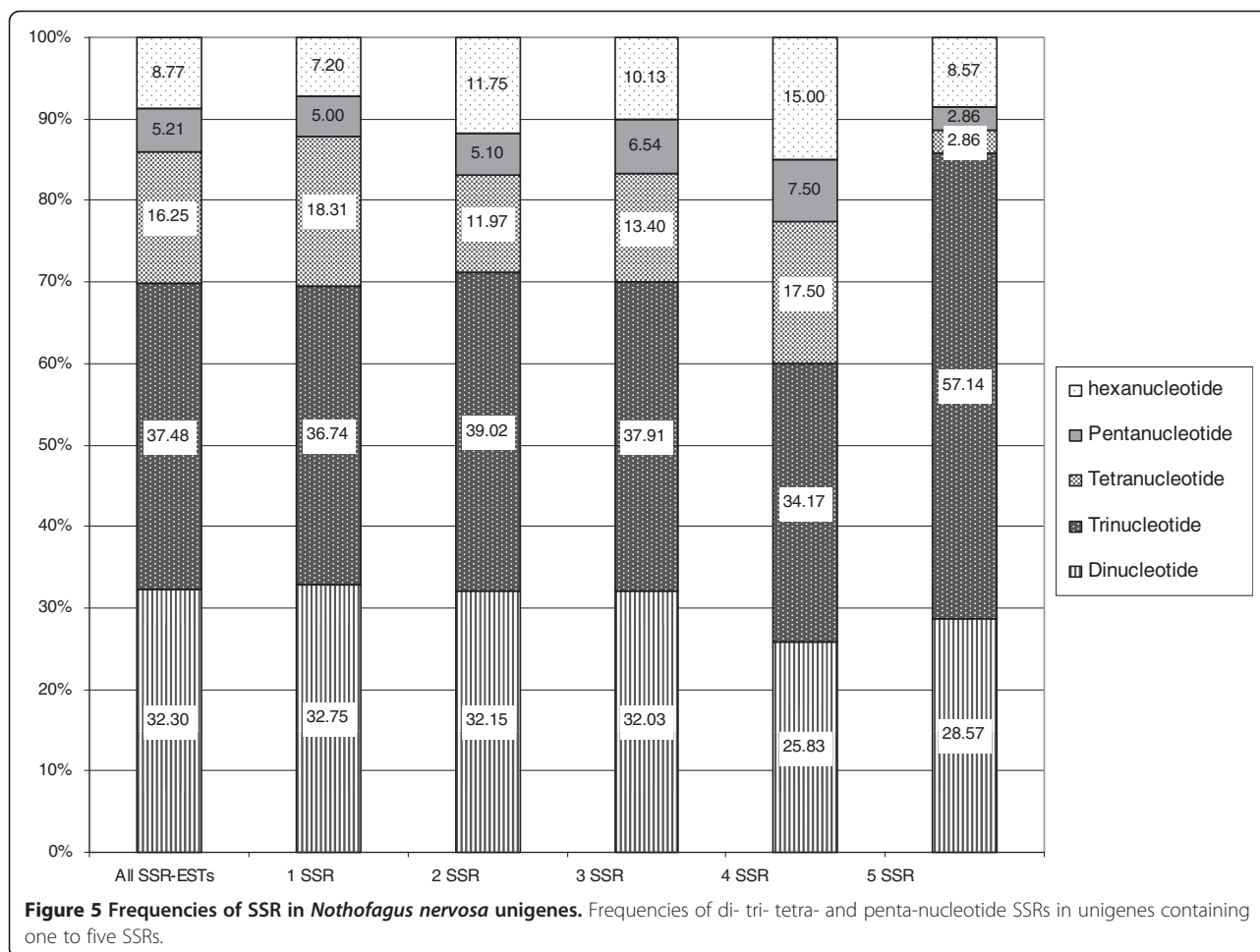
SSRs (microsatellites) motives as potential molecular markers in the *Nothofagus* unigene collection.

The criteria used for SSR selection based on the minimum number of repeats was as follows: five for dinucleotide, four for trinucleotide, three for tetranucleotide and three for penta and hexanucleotide motives. These settings resulted in the identification of 3,821 putative SSRs within 24,886 unigenes i.e. SSR frequency of 15% considering multiple occurrences in a same unigene element. This was similar than that reported in oak 19% by Durand [35] and somewhat lower than 24%, estimated by Ueno [30]. A total of 3,048 (12%) unigenes contained at least one SSR, and 2,517 SSRs (66%) had sufficient flanking sequences to allow the design of appropriate unique primers. Information on the unigene identification (ID), marker ID, repeat motive, repeat length, primer sequences, positions of forward and reverse primers, and expected fragment length are included in Additional file 2.

#### **Characterization of microsatellite motives**

As expected, the most frequent type of microsatellite corresponded to trimeric (37.4%) and dimeric motives (32.3%), being tetra-, penta- and hexanucleotide repeats present at much lower frequencies (16.3%, 5.2% and 8.8% respectively, Figure 5). Similar results were found in oak [30] (36.6% for trimeric and 36.2%, for dimeric motives) with the minimum repeat number of five and four for di- and tri-microsatellites, respectively.

SSR motif combinations can be grouped into unique classes based on DNA base complementarities. For example, dinucleotides were grouped into the following four unique classes: AT/TA; AG/GA/CT/TC; AC/CA/TG/GT and GC/CG. Thus, the numbers of unique



classes possible for di-, tri- and tetra-nucleotide repeats are 4, 10, and 33, respectively [36,37]. The AG/CT group was the predominant class (56.2%) of the dinucleotide repeats, whereas AT (29.2%), AC (14.5%) and CG (0.1%) groups were less represented. The frequency of AG was similar to the highest value reported by Kumpatla [38] (14.6%–54.5% of the total SSRs observed in 55 dicotyledonous species) but lower than that found in Oak (70.5%) [30] and eucalypts (91%) [39].

The most frequent trimeric SSR motives were the AAG/CTT (27.8%), ATG/CAT (15.2%), AGC/GCT (12.6%) and AGG/CCT (11.6%), similar to the first category found in oak (26.8%) [30]. Within tetrameric motives, AAAT repeat was found to be the most abundant (32.9%), followed by AAAG (22.7%) and AACA (11.6%).

The topography of SSR distribution was analyzed for SSR presence within UTRs and coding sequence regions. About 45% of the SSR sequences were inside ORF sequences. Most trinucleotide repeats were found in ORFs (52%), while dinucleotides were more frequent in the UTRs (40%), similar to those reported in oak [30] and pines [40]. It is expected that tri- and hexanucleotide

repeats would occur more frequently than other motifs in coding sequences. Such dominance of triplets over other repeats in coding regions may be explained on the basis of the selective disadvantage of non-trimeric SSR variants in coding regions, possibly causing frame-shift mutations [41].

#### Validation of the predicted microsatellite markers

Seventy three microsatellites were selected according to their sequence length, GC content and functional annotation related to abiotic stress category.

From these, 57% were located in coding regions. The 73 loci were tested for successful PCR amplification in six individuals. All of them were effectively amplified validating the quality of the assembly and the utility of the SSRs produced. A similar research carried using Illumina sequencing technology in sesame showed that about 90% primer pairs successfully amplified DNA fragments [42]. On the other side, the rate of SSR validation was lower (64.9%) when the marker mining was done using EST produced by Sanger technology [39] possibly because of low-quality EST sequences, and/or



primer sequences derived from chimerical cDNA clones.

About 20% (14 SSR) of the tested *Nothofagus* SSRs were polymorphic and showed at least one individual that differed in allelic composition.

This relative low percentage of polymorphic loci could be explained because of the small sample size tested (six seedlings), in contrast to the 46% found in *E. globulus* [39] evaluated in 8 samples, and the 80% found in sesame [42] assayed in 24 samples.

Nine of the polymorphic SSR found in this work were located within predicted ORF and seven had repeat motives multiple of three (Table 2), according to their presence in coding regions [41].

## Conclusions

The transcriptome database obtained and characterized here represents a major contribution for *N. nervosa* genomics and genetics. It will be useful for discovering genes of interest and genetic markers to investigate functional diversity in natural populations, and as well as conduct comparative genomics studies in southern beeches taking advantage of their remarkable ecophysiological differences. This work highlights the utility of transcriptome high performance sequencing as a fast and cost effective way for obtaining rapid information on the coding of genetic variation in *Nothofagus* genus. This study allowed us to: (i) obtain 146,267 transcript raw reads and 24,886 unigene sequences from *N. nervosa*, (ii) identify putative function in 15,497 unigenes for the genus that potentially represent 50% of *N. nervosa* transcriptome, (iii) identify 756 putative stress-response genes (21 non described in Fagaceae), (iv) discover 2,517 SSRs with designed primers and (v) detect 14 polymorphic SSR related to stress response.

## Methods

### RNA preparation and cDNA library synthesis

Total RNA was prepared by the method of Chang and collaborators [43] from leaves of one single seedling. One gram of fresh tissue was used, ground to a fine powder under liquid nitrogen. Then, after 2 extractions with chloroform, RNA was precipitated with LiCl<sub>2</sub>, extracted again with chloroform and finally precipitated with ethanol. The resultant RNA was resuspended in 50 µl of DEPC treated water. RNA was quantified using a Nanodrop 1,000 spectrophotometer and the quality was measured with a 2,100 Bioanalyzer (Agilent Technologies Inc.) Total RNA isolated was purified using the Poly (A) Purist kit (Ambion) and the quality assessed with a 2,100 Bioanalyzer (Agilent Technologies). cDNA was synthesized using cDNA Kit (Roche) and used to construct a shotgun library for pyrosequencing technology (Roche). *Nothofagus* cDNA library was subjected to

a 1/3 of plate production run on the 454-GS-FLX sequencing instrument. 454 library and sequencing was conducted at INDEAR (Rosario Biotechnology Institute, Rosario, Argentina).

### Transcript assembly and analysis

After removing low quality sequences, filtering for adaptors and primers, curated raw 454 read sequences were assembled into contigs, isotigs and isogroups using Newbler Assembler software 2.5p1 (Roche, IN, USA). Reads identified like singletons (i.e., reads not assembled into isotigs) after assembly, were subjected to CD-HIT-454 clustering algorithm using a sequence identity cut-off of 90%, which eliminates redundant sequences or artificial duplicates.

BLASTX (e-value cut off  $\leq 10e^{-10}$ ) searches were performed against Viridiplantae protein database first, then the sequences with no hits were used to perform a successive BLASTX against the NCBI *nr* protein database in order to make an assessment of the putative identities of the sequences. Also we performed a pairwise alignment using BLAT (dnax) against the Fagaceae family sequences to search expressed sequence exclusively for *N. nervosa*. Annotation and mapping routines were run with BLAST2GO, which assigns Gene Ontology (GO; <http://www.geneontology.org>) annotation, KEGG maps (Kyoto Encyclopedia of Genes and Genomes, KASS) and an enzyme classification number (EC number) using a combination of similarity searches and statistical analysis [29].

To search for chloroplast sequences we performed BLASTN and TBLASTX (BLASTN  $e^{-50}$ , TBLASTX  $10e^{-10}$ ) by similarity (with and without translation) to 109 chloroplasts (nt and aa) from chloroplast genome data base (<http://chloroplast.cbio.psu.edu/organism.cgi>).

### SSR discovery

In order to identify SSRs for all possible combinations of dinucleotide, trinucleotide, tetranucleotide and pentanucleotide repeats the SSR webserver (GDR) was run ([http://www.rosaceae.org/bio/content?title=&url=/cgi-bin/gdr/gdr\\_ssr](http://www.rosaceae.org/bio/content?title=&url=/cgi-bin/gdr/gdr_ssr)). The same tool used GETORF algorithm (EMBOSS Package) to selected the longest ORF as the putative coding region, and Primer 3 (v.0.4.0) [44] to design primer pairs.

The presence of expressed repetitive DNA was performed using the BLASTN (e-value cut off  $\leq 10e^{-10}$ ) searches against all Viridiplantae Repbase and CENSOR [45], a software tool that screens query sequences against a reference collection of repeats, and "censors" (masks) homologous portions with masking symbols, as well as generating a report classifying all found repeats.

**Table 2 Polymorphic SSRs primer pairs derived from *N. nervosa unigenes***

ID name	Locus	Repeat motif	ORF	Forward and Reverse Primers	Amplicon length observed	BLASTX, seq description	Seq Length (bp)	Sim mean (%)	GO terms related to stress response
isotig00192	INTANOT1	(tct)5	Y	F: CCAGATGGGTTTTGCTTGT R: GACGATGAAGACGATGAGC	148	heat shock protein 81-1	2309	97.2	response to stimulus
isotig00230	INTANOT2	(tcg)5	N	F: TTTCCAAACGGTTCAGAAG R: AACGAGAAGGATGTTTCCA	120	af367280_1at3g56860 t8m16_190	1229	76.6	response to stress
isotig00551	INTANOT3	(tcattt)3	Y	F: CCGATGTGATCGATAGGCTT R: CATGTCCCCAGTTCACCTCT	204	ac005850_9highly similar to mlo proteins	1759	77.5	defense response to fungus
isotig00597	INTANOT4	(ta)6	N	F: AAAACACCACCAACCCAAA R: CTTTGCCACGGCAACTAAAT	197	dnaj heat shock n-terminal domain-containing protein	1516	78.3	response to stimulus
isotig01207	INTANOT5	(tct)7	N	F: CTCGAAGACGCTACCAGACC R: TCCTGGGTTTTGCATATTGG	280	af214107_1 -like protein	748	79.3	response to stimulus
isotig01232	INTANOT6	(atc)4	Y	F: CGTTTCCCTTTAGCTGATGC R: GCTGAGTTAGCAATGGAGGC	173	aldh6b2 3-chloroallyl aldehyde dehydrogenase methylmalonate-semialdehyde dehydrogenase oxidoreductase	741	96.8	response to stress
GR7D2IN01BK031	INTANOT7	(ag)5	N	F: GACGACATCGTCCGAGTTT R: GTTAATCCCTCTCTCCTCAT	241	f-box family protein	536	75.4	response to heat
GR7D2IN01CGQUT	INTANOT8	(ccgaaa)3	Y	F: CTCCTCAACACCTCCAAA R: ATTCAAGTGGTCTTGCTG	236	mitogen-activated protein kinase kinase	518	90.5	response to osmotic stress
GR7D2IN01EMGE0	INTANOT9	(ct)8	N	F: CCGGCTACCTGTTTGTTTTA R: TTCCTTGATGATTCTTCGGG	155	at1g78870 f9k20_8	507	100.0	response to metal ion
GR7D2IN02FPPC7	INTANOT10	(ggt)6	Y	F: AAAATTGCTGTGAGGGTGG R: CCTGAATCACCAGACCGAC	117	af361609_1at1g27760 t22c5_5	529	87.9	response to osmotic stress
GR7D2IN02GFAUT	INTANOT11	(gaa)4	Y	F: ATCCCAATCTTCCCAATC R: AATTCTGTCCGCTTTGGCTA	115	salt overly sensitive 1	315	78.5	response to reactive oxygen species; response to osmotic stress
GR7D2IN02GR6NZ	INTANOT12	(at)5	Y	F: TCTTGTGGCAAGTGCTTGAG R: ACTATCCTCACCGTTGCCTG	285	win2_soltu ame: full = wound-induced protein win2 flags: precursor	472	94.0	defense response
GR7D2IN02HOKOI	INTANOT13	(tc)5	Y	F: ATATCCTGGAAATGCTTGCG R: TAAACGATCTTCGGAATGGC	124	exec1_arath ame: full = protein executor chloroplastic flags: precursor	469	71.7	response to reactive oxygen species
GR7D2IN02HWXOR	INTANOT14	(tgg)8	Y	F: AGGAGCTAAATGGGCGTAA R: CACCACCACCACCAAGAA	260	glycine-rich rna-binding protein	452	86.5	response to stress

Included are ID names, primer names, motive and number of repeats, position in ORF, sequence of forward and reverse primers (5'3'), amplicon length (bp), BLASTX similarity matches (Putative Function), Sequence length, Similarity Mean (%), GO terms related to stress response.

### SSR validation

For validation of SSR primers, total DNA was extracted from young leaves of six *N. nervosa* seedlings using the Dneasy Plant mini kit (Qiagen), following the manufacturer's instructions.

Regular primers at small scale were synthesized (AlphaDNA, Montreal, CA, USA) and used for PCR amplification. PCR reactions consisted of 20 ng total, 0.25  $\mu$ M of each primer, 3 mM MgCl<sub>2</sub>, 0.2 mM of each dNTP, 1X PCR buffer and 1 U Platinum Taq polymerase (Invitrogen). All polymerase chain reactions amplifications were performed with the following conditions: denaturation step of 2 min at 94°C, a regular touchdown PCR ranging from 60°C to 50°C (except INTANOT14 (annealing at 55°C)) with 28 cycles at the touchdown temperature of 50°C according to: 45 s at 92°C, 45 s at 50°C and 45 s at 72°C. The final extension step was of 10 min at 72°C. Samples were mixed with denaturing loading buffer, incubated for 5 min at 95°C, and separated on a 6% polyacrylamide gel. Amplification products were stained using the DNA silver staining procedure of Promega, USA, following the manufacturer's instructions. Details of primers sequences, SSR location and amplicon sizes are described in Table 2.

### Additional files

**Additional file 1: KEGG Pathway maps.** This table provides information on the enzymes putatively encoded by the RNA sequences, based on homology prediction and their associated pathways. This includes KEGG maps, enzyme names, and sequences ID.

**Additional file 2: In silico SSRs derived from *Nothofagus* leaf transcriptome (24,886 unigenes).** The data describe the 3,821 SSR: Included are unigenes names, marker ID, Sequence Length (bp), SSR description (# SSRs per seq, repeat length, motif, # Repeats, SSR position (start, stop)), ORF definition (start, stop, SSR in ORF), primers description (sequence of forward and reverse primers), expected product size (bp), similarity matches, E value, similarity mean, #GO, GO terms, Enzymes codes.

### Competing interests

The authors declare that they have no competing interests.

### Authors' contributions

SLT organized the research, provided funds, contributed to RNA extraction, data analysis and wrote manuscript. MR carried out all bioinformatics analysis and contributed to draft the manuscript. MFP contributed to RNA extraction and SSR validation. PF contributed to RNA extraction and manuscript revision. CVA contributed to analyses involving BLAST, SSR characterization and contributed to draft the manuscript. PM provided the biological material for transcriptome sequencing and manuscript revision. SG assisted the bioinformatics analysis. MMA contributed to write the project and manuscript revision. LAG conceived this study and contributed to conceptual planning of the research. HEH conceived this study, assisted in the interpretation of the results and helped to draft the manuscript. NBP participated in the design of the study, supervised the bioinformatic analysis and reviewed the manuscript. SNMP provided funding, was involved in research design, SSR data analysis and contributed to draft and revision of the manuscript. All authors approved the final manuscript.

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