Genomic sequencing and microsatellite marker development for *Boswellia papyrifera*, an economically important but threatened tree native to dry tropical forests

A. B. Addisalem¹²³, G. Danny Esselink¹, F. Bongers² and M. J. M. Smulders¹*

¹ Wageningen UR Plant Breeding, Wageningen University and Research Center, PO Box 386, NL-6700 AJ Wageningen, The Netherlands
² Center for Ecosystem Studies, Forest Ecology and Forest Management Group, Wageningen University and Research Center, PO Box 47, NL-6700 AA Wageningen, The Netherlands
³ Wondo Genet College of Forestry and Natural Resources, PO Box 128, Shashemene, Ethiopia

Received: 11 July 2014; Accepted: 8 December 2014; Published: 7 January 2015

Associate Editor: Kermit Ritland

Citation: Addisalem AB, Esselink GD, Bongers F, Smulders MJM. 2015. Genomic sequencing and microsatellite marker development for *Boswellia papyrifera*, an economically important but threatened tree native to dry tropical forests. AoB PLANTS 7: plu086; doi:10.1093/aobpla/plu086

Abstract. Microsatellite (or simple sequence repeat, SSR) markers are highly informative DNA markers often used in conservation genetic research. Next-generation sequencing enables efficient development of large numbers of SSR markers at lower costs. *Boswellia papyrifera* is an economically important tree species used for frankincense production, an aromatic resinous gum exudate from bark. It grows in dry tropical forests in Africa and is threatened by a lack of rejuvenation. To help guide conservation efforts for this endangered species, we conducted an analysis of its genomic DNA sequences using Illumina paired-end sequencing. The genome size was estimated at 705 Mb per haploid genome. The reads contained one microsatellite repeat per 5.7 kb. Based on a subset of these repeats, we developed 46 polymorphic SSR markers that amplified 2–12 alleles in 10 genotypes. This set included 30 trinucleotide repeat markers, four tetranucleotide repeat markers, six pentanucleotide markers and six hexanucleotide repeat markers. Several markers were cross-transferable to *Boswellia pirrotae* and *B. popoviana*. In addition, retrotransposons were identified, the reads were assembled and several contigs were identified with similarity to genes of the terpene and terpenoid backbone synthesis pathways, which form the major constituents of the bark resin.

Keywords: Conservation genetics; resin; SSR; terpene biosynthesis; terpenoid; tropical dry forest.

Introduction

Conservation genetics is an essential tool in conservation biology, providing insights into the genetic structure of endangered species, the dynamics of genetic variation over space and time, and the evolutionary history of populations. Microsatellite markers are highly informative DNA markers that are widely used in conservation genetics research. Next-generation sequencing enables efficient development of large numbers of SSR markers at lower costs.

*Corresponding author’s e-mail address: rene.smulders@wur.nl

Published by Oxford University Press on behalf of the Annals of Botany Company.

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted reuse, distribution, and reproduction in any medium, provided the original work is properly cited.
and markers based on them are frequently transferable across related species (Chase et al. 1996; Smulders et al. 1997, 2001; Brondani et al. 1998; Pastorelli et al. 2003; Tuskan et al. 2004; Selkoe and Toone 2006; Allan and Max 2010; Fan et al. 2013).

Recently, next-generation sequencing technologies have simplified generating large amounts of sequences at affordable cost, thus facilitating the development of molecular markers, including SSRs and single-nucleotide polymorphisms (SNPs) (Edwards et al. 2011; Ekblom and Galindo 2011; Castoe et al. 2012; Smulders et al. 2012; Lance et al. 2013; Vukosavljev et al. 2015), as well as chloroplast sequences for phylogeographical studies (Van der Merwe et al. 2014). The development of markers has thus become feasible also for species for which no prior sequence information exists (Smulders et al. 2012), including understudied but economically important crops (Zalapa et al. 2012).

Marker development can be based on short-length sequences from genomic DNA sequences or cDNA (RNA-seq). Both sets of reads are useful, but they differ with regard to further data mining. RNA-seq data can be de novo assembled into a (partial) transcriptome (Yang and Smith 2013) with some caveats, partly related to the assembler used (Shahin et al. 2012). A common denominator appears to be that multiple assemblers need to be compared (Nakasugi et al. 2014), but the final result can be compared with the transcriptome of other species. In contrast, it is not straightforward (Vicedomini et al. 2013) to assess the quality of a de novo assembly of short reads of genomic DNA from a species for which no prior sequence information is available, especially if the genome is large and contains many repeats, and the species is heterozygous or even polyploid. Nevertheless, many studies are based on genomic DNA, as it is easier to extract DNA from dry material of wild species collected in the field (on silica gel) than to try to extract good quality RNA from fresh samples or from samples specifically prepared for RNA extraction. What additional information can be reliably extracted from a single library of short reads of genomic DNA is an open question.

Boswellia papyrifera is currently the number one frankincense-producing tree species in the world (Coppen 2005). Frankincense is an aromatic resinous gum exudate produced from the bark of trees. Its economic value in the world market stems from its use as an ingredient in pharmaceuticals, cosmetics and as a church incense (Groom 1981; Tucker 1986; Lemenih and Teketay 2003). In Ethiopia, besides its value in the national economy, it has a significant contribution in the local livelihoods, providing up to one-third of annual household income, especially in the northern regions of the country (Lemenih et al. 2003, 2007; Woldeamanuel 2011).

The population size of B. papyrifera is declining in Ethiopia (Abiyu et al. 2010; Groenendijk et al. 2012; Tolera et al. 2013), Eritrea (Ogbazghi et al. 2006) and Sudan (Abtew et al. 2012). Little or no tree regeneration occurs in its natural range and mortality of adult trees increases. Despite its endangered status and economic importance, very few conservation efforts exist and none are supported by genetic information. The later situation results because genetic markers for the species have not been developed.

In the present study, we applied the Illumina paired-end sequencing technology to sequence genomic DNA of B. papyrifera with the goal of identifying microsatellite repeats and developing SSR markers. The reads were also assembled into the first genomic resource for this species, and we present a couple of structural and functional analyses on them.

Methods

Plant material

Boswellia papyrifera is one of the six Boswellia species that grow in various parts of Ethiopia. The B. papyrifera genotype used for Illumina paired-end sequencing was collected from a natural population at Kafa Humera Wukdet (14.05265N latitude; 37.13078E longitude) in north-west Ethiopia. Young leaves were collected from growing shoot tips of the plant and preserved in silica gel while in the field and during transportation to the laboratory for DNA extraction. A genomic DNA library for Illumina paired-end sequencing was prepared from 4 μg of DNA following the PCR-based gel-free illumina TruSeq DNA sample prep protocol and sequenced as 2 × 100 nt paired-end reads on an Illumina HiSeq at Greenomics, Wageningen UR, Wageningen, the Netherlands.

Plant material for SSR marker development

For testing of the SSR loci a set of 12 genotypes were used. Ten of the genotypes represented populations of B. papyrifera collected from 10 different regions of Ethiopia. Two genotypes of Boswellia pirotae and B. popovina were included for testing the cross-transferability of the markers to closely related species. The B. pirotae sample was from the northwestern part of Ethiopia. Boswellia popovina is endemic to Socotra Island, Yemen, and the dried leaf sample was obtained through the Edinburgh Royal Botanical Garden, UK.

DNA extraction

Total DNA was isolated from silica-dried young leaves following the cetyltrimethylammonium bromide protocol of Fulton et al. (1995). As large amounts of phenolic compounds were expected because of the resin content in the
leaves, the protocol was modified by the addition of 2 % pvp-40 to the extraction buffer and 1 % mercaptoethanol to the microprep buffer of Fulton et al. (1995), added immediately before use. The extraction was followed by purification steps using DNeasy (Qiagen, Venlo, The Netherlands) according to Smulders et al. (2010). DNA yield and quality were visually assessed on a 1 % agarose gel.

Sequence filtering

The raw reads were error-corrected using musket (Liu et al. 2012b). This error-corrected set was used for the repeat assembly. Prinseq-lite 0.20.04 (Schmieder and Edwards 2011) was used for quality control and filtering of reads (minimum read length of 50 nt, minimum average base quality of 25, maximum ambiguous nt (N) of 1) after which the data were used for SSR mining. After low complexity trimming (minimum DUST score of 7 for removal of low complexity reads and removal of duplicate reads, also with Prinseq-lite), paired-end reads with overlapping sequences were connected using connecting overlapped pair-end (Liu et al. 2012a) in the full mode. Reads were filtered for chloroplast sequences by mapping the reads against the closest chloroplast genome available, which is one of Citrus sinensis, using bowtie2 (Langmead and Salzberg 2012, settings -D 20 -R 3 -N 1 -L 20 -i S,1,0,50 -a).

Repeat analysis

Reads from the highly repeated fraction of the genome were extracted and assembled using RepARK (REPetitive Assembly of Repetitive k-mers; Koch et al. 2014). The motifs present in the repetitive contigs were counted and analysed by blastn (e-value 1e-5) against Repbase v19.08 (database of repetitive DNA elements, Jurka et al. 2005).

Assembly and annotation

A de novo draft assembly was created from the filtered reads using SOAPdenovo 2.21 (Li et al. 2010, settings -K 41 -M 3 -d 4). The gaps emerging during the scaffolding process by SOAPdenovo were closed using GapCloser (vs. 1.12). The contigs >1000 bp of the draft assembly were analysed and functionally annotated using Blast2GO (Conesa et al. 2005).

SSR mining and design of primers

Five million of the filtered but not assembled reads were analysed with PAL_FINDER 0.02.03 (Castoe et al. 2012) to identify SSRs using slightly adjusted criteria: at least six contiguous repeat units for dinucleotide repeats, four for tri- and tetrancleotide repeats and three for pentanucleotide repeats (Castoe et al. 2012) and six units for trunucleotide repeats. Following Castoe et al. (2012) the reads with multiple SSR loci were considered a ‘compound’ repeat if the SSRs had a different repeat motif, but a ‘broken’ repeat if the SSRs had the same motif. Reverse-complement repeat motifs (e.g. TG and CA) and translated or shifted motifs (e.g. TGG, GTG and GGT) were grouped together, so that there were a total of four unique dinucleotide repeats, 10 unique trinucleotide repeats and so on.

A subset of over 70 000 trinucleotide to hexanucleotide repeat-containing reads was used to further screen potentially amplifiable SSR loci (PALS: loci for which PCR primers could be designed. Primer designing followed the default parameters specified in Primer3 (Rozen and Skaletsky 2000). The reads were then screened for differences in lengths of those sequences that contained these primers (as in Vukosavljev et al. 2015). At these loci the sequenced plant may be heterozygous, thus indicating that the locus is polymorphic. These formed the group of potentially polymorphic loci.

SSR loci amplification and analyses of polymorphism

PCRs were performed in a total volume of 10 µL reaction mixture containing 4 µL 2 ng µL−1 DNA, 5 µL MP mix from Qiagen kit, 0.8 µL (2 µM) universal fluorescent-labelled primer and 0.2 µL mix of the forward and reverse primers. The fluorescent labelling method described in Schuelke (2000) was adapted to label the primers for analyses of the PCR products with a laser detection system. For this the forward primers were labelled with a universal M13 sequence (AA CAGGTATGACCATGA) at the 5′ end while the reverse primers were tailed with GTTT at their 5′ end according to Brownstein et al. (1996) to reduce stutter bands (both tailing sequences are not shown in the sequences in Table 1). A thermal cycling profile was set at 15 min of initial denaturation at 95 °C, followed by 30 cycles of 30 s denaturation at 94 °C, 45 s annealing at 56 °C and 45 s extension at 72 °C. This was followed by additional eight cycles with 53 °C annealing temperature to facilitate the annealing of the fluorescent dye-labelled M13 primer, and a final extension step of 10 min at 72 °C. After amplification 10 µL water was added. Fluorescently labelled amplicons were resolved on a 4200 or 4300 Licor DNA analyser.

Results

Next-generation sequencing

Genomic DNA of one B. papyrifera individual was sequenced in order to obtain a library to mine for microsatellite repeats. One lane on an Illumina HiSeq produced 143 458 368 raw reads. Based on k-mer counts, the estimated genome size of B. papyrifera was 705 Mb, sequenced at 36× coverage. After error correction and filtering reads
Table 1. Forty-six polymorphic microsatellite markers developed for *B. papyrifera* and their cross-transferability to *B. pirrotae* and *B. popoviana*.  

| Name | Primer sequence (5′ → 3′) | Repeat motif | A<sup>1</sup> | Allele size range (bp) | Quality (Smulders et al. 1997) | Ho<sub>2</sub> based on 10 *B. papyrifera* genotypes | Other Boswellia species<sup>3</sup> |
|------|------------------------|--------------|--------|---------------------|-------------------------------|---------------------------------|-------------------------|
| Bp01 | F:TTGTTAAGGCTTTTCCTCCT R:GTTGCTTATCTTTGGCTGAG | (AAG)<sub>6</sub> | 4 | 119 – 134 | 2 | 0.34 | Br = het |
| Bp02 | F:TGGAGGATTTACACCTTGTTT R:TCCTGCTCTTCTTCTTATT | (ATT)<sub>13</sub> | 7 | 195 – 219 | 2 | 0.78 | Br = hom |
| Bp03 | F:ATGGGGAAGGTAAAGAGCT R:CTGCAACAACAAAGTAAAGC | (ATC)<sub>6</sub> | 3 | 123 – 129 | 1 | 0.1 | Br = het |
| Bp04 | F:TAATCAACTTTTTGTTTGC R:CAATTGAGCTTCTCTCAAC | (TTC)<sub>8</sub> | 2 | 182 – 197 | 3 | 0.2 | Br = het |
| Bp05 | F:GGAGCAGGATCTTGTATGT R:AAAGATCTCTGTTGTTATT | (AAC)<sub>7</sub> | 5 | 232 – 250 | 1 | 0.8 | Br = hom |
| Bp06 | F:GACTTCTCAGTGACTAGGAGC R:ACATGAAAATTGAAGCAC | (ATT)<sub>9</sub> | 8 | 263 – 297 | 1 | 0.5 | Br = het |
| Bp07 | F:GAAAACCTTGGGTTTGTGT R:TCATCTCCTGACATATCATT | (ATT)<sub>8</sub> | 7 | 284 – 293 | 1 | 0.34 | Br = hom |
| Bp08 | F:TTCGCTTGTGTTGTTCAGCA R:GGATGCAAGAAAGGAGAGG | (ATT)<sub>6</sub> | 6 | 207 – 213 | 2 | 0.11 | Br = no ampl |
| Bp09 | F:TTGATATATTCTTGGAGCA R:AATTAGATGGCTCTGGTAA | (ATT)<sub>11</sub> | 7 | 292 – 331 | 1 | 0.78 | Br = no ampl |
| Bp10 | F:CCTCGGCAAATCTACAGAG G:GTACGACGAAATAGGAGGA | (TTC)<sub>6</sub> | 4 | 197 – 213 | 1 | 0.11 | Br = het |
| Bp11 | F:AGAGAATTCCTAAGAGGAGA R:TCTACATAGCAGGACCACT | (TTC)<sub>9</sub> | 6 | 284 – 307 | 1 | 0.78 | Br = hom |
| Bp12 | F:ACCCATGATAAGAGTTCCA R:GAGAACGCCGTGTGTTA | (ATT)<sub>10</sub> | 7 | 238 – 302 | 2 | 0.56 | Br = het |
| Bp13 | F:AATATTCCACACAGGAGAT R:CAAGAAGACTACAGATTGAATG | (ATT)<sub>7</sub> | 3 | 227 – 239 | 1 | 0.22 | Br = hom |
| Bp14 | F:GCGAAATCTTGTAGCTAC R:ATGACATCCATGCTGAACC | (ATT)<sub>15</sub> | 8 | 198 – 253 | 1 | 0.44 | Br = het |
| Bp15 | F:TCATGCCTCTGCTTCTCA R:AAACTCCAGGCTGACTACAC | (ATT)<sub>10</sub> | 7 | 301 – 337 | 1 | 0.78 | Br = het |
| Bp16 | F:AAAACTTGGTCTCTTCCTCA R:TCAGAAGACCTCTACCTCA | (TCC)<sub>11</sub> | 2 | 218 – 221 | 1 | 0.33 | Br = hom |
| Bp17 | F:AGCAAATTTCAAGAGGACA R:TGCACATACAGAGTACCC | (TTC)<sub>11</sub> | 6 | 200 – 215 | 1 | 0.4 | Br = no ampl |
| Bp18 | F:TTTTTGTTCTGAGGATGGG R:GAAACTTTGAAATTTGTTA | (ATT)<sub>6</sub> | 2 | 221 – 262 | 2 | 0.67 | Br = hom |

Continued
### Table 1. Continued

| Name | Primer sequence (5'→3') | Repeat motif | A | Allele size range (bp) | Quality | Ho² based on 10 B. papyrifera genotypes | Other Boswellia species³ |
|------|-------------------------|--------------|---|----------------------|---------|----------------------------------------|-------------------------|
| Bp19 | F:GTGCCAGAATTCAGGTATGT R:GGTTGGAATGCCAATAT | (TTC)13 | 5 | 287–321 | 2 | 0.1 | Br = het |
| Bp20 | F:TTCCCTGTGACTTTGTTGAGA R:GAACCATCATGCAATTAGT | (TTC)15 | 10 | 227–266 | 2 | 0.5 | Br = het |
| Bp21 | F:CAGAGTTAATAATATAAGTAGCAGCA R:CTATGCTTCTAATCTTAGAAAAGTGGG | (TTC)16 | 12 | 117–299 | 1 | 0.6 | Br = hom |
| Bp22 | F:GAAAACCCTTTGCAAGAAGG AGCACCCTTTCAAAATCA | (TTC)17 | 11 | 237–307 | 1 | 0.7 | Br = hom |
| Bp23 | F:GGAATTGGTTGCTAGTAATCC R:TAAGGCCCAAGAAATGGA | (TTC)18 | 11 | 224–266 | 2 | 0.8 | Br = het |
| Bp24 | F:TATTTGTGACTTCAAGATGGGG GACCTTTGACTTTTG | (TTC)19 | 12 | 241–251 | 1 | 0 | Br = hom |
| Bp25 | F:ATCATCATCAGTGAAAGACC R:AGTCTGTTTCTGCTTCC | (TTC)20 | 4 | 261–279 | 1 | 0.22 | Br = hom |
| Bp26 | F:AAATCATGTGGTCTAATGG R:TGGCCAATGCAATTAATGGA | (TTC)21 | 3 | 235–247 | 1 | 0.34 | Br = hom |
| Bp27 | F:CTCTAGATGACTTTAGGATGG R:AATATATCGCTAAACCTGG | (TTC)22 | 2 | 240–246 | 1 | 0.25 | Br = no ampl |
| Bp28 | F:CAATACCTTGTGTATTCC R:AATGAGCAATAATGACAGGG | (TTC)23 | 4 | 262–272 | 1 | 0.14 | Br = het |
| Bp29 | F:ATATCTAGAGACTTGGGCC R:TTACCAAATGAGCTAAGACG | (TTC)24 | 6 | 249–264 | 1 | 0.43 | Br = hom |
| Bp30 | F:ATATGCTAGAGACTTGGGCC R:TTCATGCTTGGATGAGG | (TTC)25 | 3 | 200–212 | 1 | 0.34 | Br = hom |
| Bp31 | F:AGAGAAGATAAGTAGCTTGGAC R:AGGGAAGAGTAGCTTGG | (TTC)26 | 4 | 277–307 | 2 | 0.75 | Br = hom |
| Bp32 | F:TCATAACTCTAAAAATGGAC R:TTTCAGCTTGGATGAGG | (TTC)27 | 3 | 144–156 | 1 | 0.11 | Br = no ampl |
| Bp33 | F:CTGTACCTCTTCTTCTTCC R:GTACTAAACCCTCCGTTCG | (TTC)28 | 2 | 171–181 | 3 | 0.33 | Br = het |
| Bp34 | F:AGAGAACATCTAAAAGATACC R:AGGATAGAGGAGAGCTTACG | (TTC)29 | 4 | 183–193 | 1 | 0.56 | Br = het |
| Bp35 | F:GGGTCTCAGCTAACCAGACC R:CACCCAGCTGAGCAGTAGGC | (TTC)30 | 2 | 224–230 | 1 | 0.1 | Br = hom |
| Bp36 | F:GGTAAACTAGAAGAGAGGATAGAAGGCAATGG | (TTC)31 | 4 | 211–226 | 2 | 0.89 | Br = hom |
| Bp37 | F:ATCTGCGTTTCTCATTCCC R:AGAACCCTCTCATTACC | (TTC)32 | 2 | 277–283 | 1 | 0.11 | Br = hom |
| Bp38 | F:GGTGGAGAAGAGAAGAGCAGG R:CATCAACTCTTCAAAATTCC | (ATC)7,8 | 5 | 243–273 | 1 | 0.22 | Br = het |
for short sequences, sequences with ambiguities (Ns) and low complexity, and excluding redundant sequences, 120 479 203 (84 %) paired-end reads and 10 851 777 single-end reads remained.

**SSR identification**

A search of SSRs in a subset of five million Illumina paired-end reads identified 170 832 reads (3.4 %) containing SSRs. In these reads, a total of 175 607 repeat loci (dinucleotide through hexanucleotide repeats) were identified, which corresponds to one SSR locus per 5.7 kb. Figure 1 shows the frequency of the top-20 repeat motifs. These include all dinucleotide motif repeats (of at least six repeat units long), of which AC and AT repeats were the most abundant. Of the trinucleotide repeats (of at least four repeat units) AAT and AAC were the most frequent, followed by TTC. Excluding the dinucleotide repeats, the remaining 70 415 SSR loci were screened for the presence of sufficient forward and reverse flanking sequences suitable to design primers. This yielded 29 886 (42 %) PALs. Further filtering of these PALs by applying the most stringent criteria aimed at selecting single-copy loci yielded 4071 potentially amplifiable SSR loci.

**Polymorphism and amplification of SSR loci**

A total of 136 SSR loci (117 randomly picked and 19 loci predicted to be potentially polymorphic as they appeared to have two different alleles in the sequence reads) were tested for amplification and degree of polymorphism in 10 randomly chosen individuals from different populations. Of the 117 randomly picked loci, 82 primer pairs amplified a high-quality PCR product, of which 37 (45 %) were polymorphic with a banding pattern that could be scored clearly (Table 1). Of the 19 primer pairs predicted to be polymorphic, 13 amplified bands of which 9 loci (69 %) were polymorphic, indicating a significantly higher rate of polymorphism ($\chi^2$ test, $P < 0.005$) compared with randomly picked loci. The final set of 46 markers included 30 trinucleotide repeat markers, 4 tetranucleotide repeats, 6 pentanucleotides and 6 hexanucleotide repeats. The number of alleles across the polymorphic loci varied between 2 and 12 with an average value of 4.8 alleles in 10 genotypes. Several of the polymorphic markers with 10–12 alleles were TTC repeats. The heterozygosity per locus ranged widely from 0.10 to 0.89 (average 0.43). It is possible that, when used in larger populations, these markers will show higher estimates of Ho, and additional alleles may be found.

As shown in Table 1, most of the SSRs successfully amplified in *B. papyrifera* and *B. popoviana* (in the latter species the amount of DNA was insufficient to test all markers). Amplification, even if it is in the same size range as the alleles in *B. papyrifera*, is not proof that the marker is

---

**Table 1. Continued**

| Name  | Primer sequence (5' → 3') | Repeat motif | A | Allele size range (bp) | Quality | Ho based on 10 *B. papyrifera* genotypes | Other Boswellia species |
|-------|---------------------------|--------------|---|------------------------|---------|----------------------------------------|-----------------------|
| Bp39  | F:TCATGGAATAAGAAACAAAAA | (ATC)8,(9)    | 8 | 247–298                | 2       | 0.6 Br = het                            |                       |
|       | R:TCTTAAACATTTCGGTCTG     |              |   |                        |         |                                        |                       |
| Bp40  | F:AAACAATAATAGTTGGCAAACA | (ATT)8,(14)  | 3 | 200–255                | 2       | 0.3 Br = hom                            |                       |
|       | R:TCCAAATGAAATCCAAAAT    |              |   |                        |         |                                        |                       |
| Bp41  | F:TGGGTAAAGTATCTAAGGGAG | (ATT)8,(9)    | 4 | 230–252                | 2       | 0.22 Br = hom                           |                       |
|       | R:CATTAGAAGGCGAAATGG     |              |   |                        |         |                                        |                       |
| Bp42  | F:TTATAACAGAGCAAATATAGG | (ATT)10,(11) | 6 | 228–264                | 2       | 0.4 Br = hom                            |                       |
|       | R:CTAAATCCGGAATTTAAGGC  |              |   |                        |         |                                        |                       |
| Bp43  | F:CCAAGGCCTACTCTCCATCA  | (TTT)6,(8)   | 6 | 272–293                | 3       | 0.89 Br = het                           |                       |
|       | R:GATGAATTGGCGTTAGATT    |              |   |                        |         |                                        |                       |
| Bp44  | F:CCATATGGGGATATAGGCTCA | (ATT)6,(7)   | 4 | 226–235                | 2       | 0.25 Br = het (out of range)           |                       |
|       | R:TTGCGCAAGAAAGAATCTAG  |              |   |                        |         |                                        |                       |
| Bp45  | F:ACAGGTTGTCTAAACAGGC   | (AACAAG)3,(4) | 3 | 281–293                | 1       | 0.67 Br = het                           |                       |
|       | R:CTTAAAGGGGACCTGGAAGG  |              |   |                        |         |                                        |                       |
| Bp46  | F:ATATTCAATTTTCTCITGAGC | (ATATT)3,(4) | 2 | 256–271                | 2       | 0.75 Br = hom                           |                       |
|       | R:TTGATTTCACAGGAACACGA  |              |   |                        |         |                                        |                       |
polymorphic, but heterozygosity (two different alleles in the expected range) is. Based on that criterion at least 19 of the 46 markers are polymorphic in *B. pirotae* and at least 8 of 33 tested are polymorphic in *B. popoviana*.

**Sequence assembly and annotation**

The Illumina reads are the first genomic resource generated in the genus *Boswellia*. The repeat fraction was assembled based on k-mer frequency. This produced 49,576 contigs of repeats that were present at least 50× (median length 139 bp, mean length 224 bp, N50 238 bp, maximum length 21,153 bp, total sum = 574 Mbp). Next, 1,533 contigs had blastn hits with RepBase, mostly with Copia (639 hits) and Gypsy (523) retrotransposons, alongside EnSpm (114), hAT (72), Satellite (29), TY (23), Harbinger (16), YPrime (14), Helitron (12) and SC TRANSP (3). Intermixed with these elements were hits to the ribosomal RNAs (LSU 56 hits, SSU 41) and also to Caulimoviridae viruses (11).

Using all data in a *de novo* assembly with SOAPdenovo, 444,927 contigs were obtained with a median of 375 bp, a mean contig length of 690 bp, an N50 of 1085 bp and a maximum contig size of 19,236 bp (total sum = 307 Mb genomic DNA sequence). The contigs ≥1000 bp were blasted against Genbank, and 65,467 were annotated with GO terms (Fig. 2; note that these are overlapping classes).

**Terpene biosynthesis genes**

*Assefa et al.* (2012) conducted a biophysical and chemical study on resins of *Boswellia* species with special emphasis on *B. papyrifera*. Using the list of identified components, eight contigs of the assembly were found, which represent part of genes of the terpene synthesis pathways, namely pinene synthase, limonene synthase (2 ×), isoprene synthase (4 ×) and gamma-terpinene synthase.

![Figure 1. The 20 most frequent SSR motifs obtained, sorted according to frequency.](image-url)

![Figure 2. Representation of ontology assignments of the *B. papyrifera* contigs. (A) The 31,086 GO terms of cellular components, (B) the 42,423 GO terms of molecular function and (C) the 54,256 GO terms of biological processes. Note that these are overlapping classes.](image-url)
Table 2. MEP/DOXP and mevalonate pathway genes found among the contigs of B. papyrifera.

| Name                  | EC no. |
|-----------------------|--------|
| **MEP/DOXP pathway**  |        |
| DXS                   | 1-Deoxy-D-xylulose-5-phosphate synthase | EC 2.2.1.7 |
| DXR                   | 1-Deoxy-D-xylulose-5-phosphate reductoisomerase | EC 1.1.1.267 |
| MDS                   | 2-C-methyl-D-erythritol-2,4-cyclodiphosphate synthase | EC 4.6.1.12 |
| HDS                   | 4-Hydroxy-3-methylbut-2-enyl diphosphate synthase | EC 1.17.7.1 |
| IDI                   | Isopentenyl diphosphate isomerase | EC 5.3.3.2 |
| GPPS                  | Geranyl-diphosphate synthase | EC 2.5.1.11 |
| GGPPS                 | Geranylgeranyl diphosphate synthase | EC 2.5.1.29 |
| CPS                   | Copalyl diphosphate synthase | EC 5.5.1.12 |
| KS                    | Kaurene synthase | EC 4.2.3.19 |
| **Mevalonate pathway**|        |
| AACT                  | Acetyl-CoA C-acetyltransferase | EC 2.3.1.9 |
| HMGS                  | Hydroxymethylglutaryl-CoA synthase | EC 2.3.3.10 |
| HMGR                  | Hydroxymethylglutaryl-CoA reductase | EC 1.1.1.34 |
| MK                    | Mevalonate kinase | EC 2.7.1.36 |
| PMK                   | 5-Phosphomevalonate kinase | EC 2.7.4.2 |
| MDC                   | Mevalonate-5-pyrophosphate decarboxylase | EC 4.1.1.33 |
| IDI                   | Isopentenyl diphosphate isomerase | EC 5.3.3.2 |
| FPPS                  | Farnesyl diphosphate synthase | EC 2.5.1.10 |

database). Table 2 lists the enzymes of the mevalonate and non-mevalonate (MEP/DOXP) pathways, the two pathways for the synthesis of terpenoid building blocks in plants, which were found among the annotation results. Two of the key enzymes of the MEP pathway, 2-C-methyl-D-erythritol-4-phosphate cytidylyltransferase (EC 2.7.7.60) and 4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol kinase (EC: 2.7.1.148), were not recognized in the set of scaffolds, but reciprocal tBlastx (at 1e-5) against these enzymes identified in Arabidopsis did reveal hits with, respectively, 3 and 2 contigs.

**Discussion**

We have developed the first set of 46 SSR markers for B. papyrifera. The markers amplified between 2 and 12 alleles in individuals from 10 different populations across Ethiopia. We based the marker development on DNA sequences from one individual. Most of the markers tested were chosen randomly, but the subset for which we assessed, from the sequence reads, that they probably had two alleles in this individual, gave a significantly higher success rate compared with the randomly chosen ones. This assessment is a technically easy screening step that would improve the efficiency of marker development in an outbreeding species, even if only sequences from one individual have been generated, as is often the case. It is probably not as efficient as a strategy that generates transcriptome sequences from multiple individuals with the specific aim of testing only those loci on gel for which polymorphisms in repeat length exist among the reads obtained from these individuals (Vukosavljev et al. 2015).

The SSR markers were developed based on a set of Illumina paired-end DNA sequence reads from young leaves of a single individual of B. papyrifera. The distribution of these reads indicated a genome size of 705 Mb. This is close to the estimate of 682 Mb for B. serrata, the only Boswellia species listed in the Kew Gardens C-value database.

Mobile elements that are present in multiple copies in the genome were analysed based on sequence homology in k-mers that occurred at high frequency (Koch et al. 2014). We have identified a series of retrotransposons, the most common being Copia and Gypsy elements. As these elements are present in large numbers, our Illumina reads probably were a sufficiently good source to determine the presence and relative frequency of various elements.

We also assembled all reads of our paired-end short-read library and obtained 307 Mb of unique sequences. The quality of this assembly is difficult to assess without...
Based on Illumina paired-end sequences, we have developed a set of polymorphic SSR markers for *B. papyrifera* and two sister species, which will be useful for studying genetic diversity within and differentiation between *Boswellia* populations. We also generated the first genomic resource in *Boswellia*.

**Accession Numbers**

Accession number in ENA/GenBank for the set of DNA sequences on which the SSR markers were developed: ERS403283.

**Sources of Funding**

This study was funded by the Netherlands’ Fellowship programme (NUFFIC).

**Contributions by the Authors**

F.B. and M.J.M.S. conceived the study. A.B.A. sampled the plants, carried out the testing and analysed the data. G.D.E. did the bioinformatics analyses. A.B.A., F.B. and M.J.M.S. wrote the paper. All authors have read and approved the submitted manuscript.

**Conflicts of Interest Statement**

None declared.

**Acknowledgements**

The authors thank Alan Forrest, the Edinburgh Botanical Garden, for providing the *B. popoviana* sample. Koen Pelm and Doret Wouters are thanked for helping in the laboratory and Robert van Loo for help in analysing the genes involved in secondary component synthesis.

**Literature Cited**

Abiyu A, Bongers F, Eshete A, Gebrehiwot K, Kindu M, Lemenih M, Moges Y, Ogbozghi W, Sterck FJ. 2010. Incense Woodlands in Ethiopia and Eritrea: regeneration problems and restoration possibilities. In: Bongers F, Tenningkeit T, eds. Degraded forest in eastern Africa: management and restoration. London, UK: Earthscan, 133–152.

Abtew AA, Pretsch J, Mohamoud TE, Adam YO. 2012. Population status of *Boswellia papyrifera* (Del.) Hochst in the dry woodlands of Nuba Mountains, South Kordofan State, Sudan. *Agriculture and Forestry* **54**:41–50.

Allan GJ, Max TL. 2010. Molecular genetic techniques and markers for ecological research. *Nature Education Knowledge* **3**:2.

Arens P, Van der Sluis Th, Van’t Westende WPC, Vosman B, Vos CC, Smulders MJM. 2007. Population differentiation and connectivity among fragmented Moor frog (*Rana arvalis*) populations in the Netherlands. *Landscape Ecology* **22**:1489–1500.

Assefa M, Dekebo H, Kassa H, Habtu A, Fitwi G, Redi-Abshiro M. 2012. Biophysical and chemical investigations of frankincense of *Boswellia popoviana* and two sister species, which will be useful for studying genetic diversity within and differentiation between *Boswellia* populations. We also generated the first genomic resource in *Boswellia*.

**Accession Numbers**

Accession number in ENA/GenBank for the set of DNA sequences on which the SSR markers were developed: ERS403283.

**Sources of Funding**

This study was funded by the Netherlands’ Fellowship programme (NUFFIC).

**Contributions by the Authors**

F.B. and M.J.M.S. conceived the study. A.B.A. sampled the plants, carried out the testing and analysed the data. G.D.E. did the bioinformatics analyses. A.B.A., F.B. and M.J.M.S. wrote the paper. All authors have read and approved the submitted manuscript.

**Conflicts of Interest Statement**

None declared.

**Acknowledgements**

The authors thank Alan Forrest, the Edinburgh Botanical Garden, for providing the *B. popoviana* sample. Koen Pelm and Doret Wouters are thanked for helping in the laboratory and Robert van Loo for help in analysing the genes involved in secondary component synthesis.

**Literature Cited**

Abiyu A, Bongers F, Eshete A, Gebrehiwot K, Kindu M, Lemenih M, Moges Y, Ogbozghi W, Sterck FJ. 2010. Incense Woodlands in Ethiopia and Eritrea: regeneration problems and restoration possibilities. In: Bongers F, Tenningkeit T, eds. Degraded forest in eastern Africa: management and restoration. London, UK: Earthscan, 133–152.

Abtew AA, Pretsch J, Mohamoud TE, Adam YO. 2012. Population status of *Boswellia papyrifera* (Del.) Hochst in the dry woodlands of Nuba Mountains, South Kordofan State, Sudan. *Agriculture and Forestry* **54**:41–50.

Allan GJ, Max TL. 2010. Molecular genetic techniques and markers for ecological research. *Nature Education Knowledge* **3**:2.

Arens P, Van der Sluis Th, Van’t Westende WPC, Vosman B, Vos CC, Smulders MJM. 2007. Population differentiation and connectivity among fragmented Moor frog (*Rana arvalis*) populations in the Netherlands. *Landscape Ecology* **22**:1489–1500.

Assefa M, Dekebo H, Kassa H, Habtu A, Fitwi G, Redi-Abshiro M. 2012. Biophysical and chemical investigations of frankincense of *Boswellia popoviana* and two sister species, which will be useful for studying genetic diversity within and differentiation between *Boswellia* populations. We also generated the first genomic resource in *Boswellia*.

**Accession Numbers**

Accession number in ENA/GenBank for the set of DNA sequences on which the SSR markers were developed: ERS403283.
**Boswellia papyfera** from north and northwestern Ethiopia. *Journal of Chemical and Pharmaceutical Research* **4**:1074–1089.

Basar S. 2005. Phytochemical investigations on *Boswellia* species. Comparative studies on the essential oils, pyrolyates and boswellic acids of *Boswellia carterii* Birdw., *Boswellia serrata* Roxb., *Boswellia frereana* Birdw., *Boswellia neglecta* S. Moore and *Boswellia rivae* Engl. PhD Thesis, University of Hamburg, Germany.

Bekana D, Kebede T, Asseffa M, Kassa H. 2014. Comparative phytochemical analyses of resins of *Boswellia* species (B. papyfera (Del.) Hochst., B. neglecta S. Moore, and B. rivae Engl.) from Northwestern, Southern, and South-Eastern Ethiopia. ISRN Analytical Chemistry 2014:374678. http://dx.doi.org/10.1155/2014/374678.

Brondani RPV, Brondani C, Tarchini R, Grattapaglia D. 1998. Development, characterization and mapping of microsatellite markers in *Eucalyptus grandis* and *E. urophylla*. *Theoretical and Applied Genetics* **97**:816–827.

Brownstein MJ, Carpenter JD, Smith JR. 1996. Modulation of non-model organisms in molecular ecology of non-model organisms. *Heredity* **76**:363–372.

Castoe TA, Poole AW, de Koning APJ, Jones KL, Tomback D. 2012. Rapid microsatellite identification from Illumina paired-end genomic sequencing in two birds and a snake. *PloS ONE* **7**:e30953.

Chase M, Kesseli R, Bawa K. 1996. Microsatellite markers for conservation and population genetics of tropical tree species. *American Journal of Botany* **83**:51–57.

Conesa A, Götz S, Garcia-Gómez JM, Terol J, Talón M, Robles M. 2005. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* **21**:3674–3676.

Coppen JJW. 2005. Overview of international trades and markets. In: Edwards CE, Parchman TL, Weekley C. 2011. Assembly, gene annotation and marker development using 454 paired-end reads. *The authors*. COPE: an accurate k-mer-based pair-end reads correction tool to facilitate genome assembly. *Bioinformatics* **28**:2870–2874.

Fulton TM, Chunwangse J, Tanksley SD. 1995. Microprop protocol for extraction of DNA from tomato and herbaceous plants. *Plant Molecular Biology Reporter* **13**:207–209.

González-Martínez SC, Krutovsky KV, Neole DB. 2006. Forest-tree populations and genomic adaptations to new environments. *New Phytologist* **170**:227–238.

Groenendijk P, Eshete A, Sterck FJ, Zuidema P, Bongers F. 2012. Limitations to sustainable frankincense production: blocked regeneration, high adult mortality, and declining population. *Journal of Applied Ecology* **49**:164–173.

Groom N. 1981. Frankincense and myrrh: a study of the Arabian incense trade. London: Longman, 285 p.

Hailey YA, Dowd SE, Decker JE, Seabury PM, Bhattrai E, Johnson CD, Rollins D, Tizard JR, Brightsmith DJ, Peterson MJ, Taylor JF, Seabury CM. 2014. A draft de novo genome assembly for the northern Bobwhite (*Colinus virginianus*) reveals evidence for a rapid decline in effective population size beginning in the late Pleistocene. *PloS ONE* **9**:e90240.

Jurka J, Kapitonov VV, Pavlicek A, Klonowski P, Kohn O, Wolichiewicz J. 2005. Repbase update, a database of eukaryotic repetitive elements. *Cytogenetic and Genome Research* **110**:462–467.

Karp A, Kresovich S, Bhat KV, Ayad WG, Hodgkin T. 1997. Molecular tools in plant genetic resources conservation: a guide to the technologies. In: IPGRI Technical Bulletin No. 2. Rome, Italy: International Plant Genetic Resources Institute.

Koch P, Platzter M, Downie BR. 2014. RepARK—de novo creation of repeat libraries from whole-genome NGS reads. *Nucleic Acids Research* **42**e80.

Lance SL, Love CN, Nunziata SO, O’Bryhim JR, Scott DE, Wesley Flynn RW, Jones KL. 2013. 32 species validation of a new Illumina paired-end approach for the development of microsatellites. *PloS ONE* **8**:e81853.

Lemenih M, Balch DJ. 2012. Fast gapped-read alignment with Bowtie 2. *Nature Methods* **9**:357–359.

Lemenih M, Teketay D. 2003. Frankincense and myrrh resources of Ethiopia: medicinal and industrial uses. *SINET Ethiopian Journal of Science* **26**:161–172.

Lemenih M, Abebe T, Mats O. 2003. Gum and Resin resources from some *Acacia*, *Boswellia*, and *Commiphora* species and their economic contributions in Liban, south-east Ethiopia. *Journal of Arid Environments* **55**:465–482.

Lemenih M, Feleke S, Tadesse W. 2007. Constraints to smallholders production of frankincense in Metema district, North-western Ethiopia. *Journal of Arid Environments* **71**:393–403.

Luo R. 2012. De novo assemblers in de novo assemblies of human genomes with massively parallel short read sequencing. *Genome Research* **20**:265–272.

Liu B, Yuan J, Yiu SM, Li Z, Xie Y, Chen Y, Shi Y, Zhang H, Li Y, Lam TW, Luo R. 2012a. COPE: an accurate k-mer-based pair-end reads connection tool to facilitate genome assembly. *Bioinformatics* **28**:2870–2874.

Liu Y, Schröder J, Schmidt B. 2012b. Musket: a multistage k-mer spectrum-based error corrector for Illumina sequence data. *Bioinformatics* **29**:308–315.

McMahon BJ, Teeling EC, Höglund J. 2014. How and why should we implement genomics into conservation? *Evolutionary Applications* **7**:999–1007.

Moran P. 2002. Current conservation genetics: building an ecological approach to the synthesis of molecular and quantitative genetic methods. *Ecology of Freshwater Fish* **11**:30–55.

Nakasugi K, Crowhurst R, Bally J, Waterhouse P. 2014. Combining transcriptome assemblies from multiple de novo assemblers in the allo-tetraploid plant *Nicotiana benthamiana*. *PloS ONE* **9**:e91776.
Nybom H, Weising K, Rotter B. 2014. DNA fingerprinting in botany: past, present, future. *Investigative Genetics* 5:1.

Ogbazghi W, Rijkers T, Wessel M, Bongers F. 2006. The distribution of the frankincense tree *Boswellia papyrfera* in Eritrea: the role of environment and land use. *Journal of Biogeography* 33:524–535.

Pastorelli R, Smulders MJM, van’t Westende WPC, Vosman B, Giannini R, Vettori C, Vendramin GG. 2003. Characterisation of microsatellite markers in *Fagus sylvatica* L. and *Fagus orientalis* Lipsky. *Molecular Ecology Notes* 3:76–78.

Primmer CR. 2009. From conservation genetics to conservation genomics. *Annals of the New York Academy of Sciences* 1162:357–368.

Rozen S, Skeatsky HJ. 2000. Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S, eds. *Bioinformatics methods and protocols: methods in molecular biology*. Totowa, NJ: Humana Press, 365–386.

Schmieder R, Edwards R. 2011. Quality control and preprocessing of metagenomic datasets. *Bioinformatics* 27:863–864.

Schuelke M. 2000. An economic method for the fluorescent labelling of PCR fragments: a poor man’s approach to genotyping for research and high-throughput diagnostics. *Nature Biotechnology* 18:233–234.

Selkoe KA, Toone RJ. 2006. Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. *Ecology Letters* 9:615–629.

Shahin A, van Gurp T, Peters SA, Visser RGF, van Tuyl JM, Arens P. 2012. SNP markers retrieval for a non-model species: a practical approach. *Molecular Research Notes* 5:79.

Smulders MJM, Bredemeijer G, Rus-Kortekaas W, Arens P, Vosman B. 1997. Use of short microsatellites from database sequences to generate polymorphisms among Lycopersicon species. *Theoretical and Applied Genetics* 94:264–272.

Smulders MJM, van der Schoot J, Arens P, Vosman B. 2001. Trinucleotide repeat microsatellite markers for black poplar (*Populus nigra* L.). *Molecular Ecology Notes* 1:188–190.

Smulders MJM, Cottrell JE, Lefèvre F, van der Schoot J, Arens P, Vosman B, Tabbeno HE, Grassi F, Fossati T, Castiglione S, Krystufek V, Fluch S, Burg K, Vornam B, Pohl A, Gebhardt K, Alba N, Agúndez D, Maestro C, Notivol E, Volosyanchuk R, Popiškova M, Bordács S, Bovenschen J, van Dam BC, Koelewijn H-P, Halfmaerten D, Ivens B, van Slycken J, Vanden Broeck A, Storme V, Boerjan W. 2008. Structure of the genetic diversity in Black poplar (*Populus nigra* L.) populations across European river systems: consequences for conservation and restoration. *Forest Ecology and Management* 255:1388–1399.

Smulders MJM, Esselink GD, Everaert J, De Riek J, Vosman B. 2010. Characterisation of sugar beet (*Beta vulgaris* L. ssp. vulgaris) varieties using microsatellite markers. *BMC Genetics* 11:41.

Smulders MJM, Vukosavljev M, Shahin A, van de Weg WE, Arens P. 2012. High throughput marker development and application in horticultural crops. *Acta Horticulturae (ISHS)* 961:547–551.

Tolera M, Sass-Klaassen U, Esthete A, Bongers F, Sterck FJ. 2013. Frankincense tree recruitment failed over the past half century. *Forest Ecology and Management* 304:65–72.

Tucker AO. 1986. Frankincense and myrrh. *Economic Botany* 40:425–433.

Tuskan GA, Gunter LE, Yang ZK, Yin Tong M, Sewell MM, DiFazio SP. 2004. Characterization of microsatellites revealed by genomic sequencing of *Populus trichocarpa*. *Canadian Journal of Forestry Research* 34:85–93.

Van der Merwe M, McPherson H, Siow J, Rossetto M. 2014. Next-Gen phylogeography of rainforest trees: exploring landscape-level cpDNA variation from whole-genome sequencing. *Molecular Ecology Resources* 14:199–208.

Vicedomini R, Vezzi F, Scalabrini S, Arvestad L, Policriti A. 2013. GAM-NGS: genomic assemblies merger for next generation sequencing. *BMC Bioinformatics* 14(Suppl. 7):S6.

Vukosavljev M, Esselink GD, Van’t Westende WPC, Cox P, Visser RGF, Arens P, Smulders MJM. 2015. Efficient development of highly polymorphic microsatellite markers based on polymorphic repeats in transcriptome sequences of multiple individuals. *Molecular Ecology Resources* 15:17–27.

Woldeamanuel T. 2011. *Dryland resources, livelihoods and institutions: diversity and dynamics in use and management of gum and resin trees in Ethiopia*. PhD Dissertation, Wageningen University, Wageningen, The Netherlands, 169p. ISBN 978-90-8585-962-8.

Yang Y, Smith SA. 2013. Optimizing de novo assembly of short-read RNA-seq data for phylogenomics. *BMC Genomics* 14:328.

Zalapa JE, Cuevas H, Zhu H, Steffan S, Senalik D, Zeldin E, McCown B, Harbut R, Simon P. 2012. Using next-generation sequencing approaches to isolate simple sequence repeat (SSR) loci in the plant sciences. *American Journal of Botany* 99:193–208.