



# Highly Informative Single-Copy Nuclear Microsatellite DNA Markers Developed Using an AFLP-SSR Approach in Black Spruce (*Picea mariana*) and Red Spruce (*P. rubens*)

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## Abstract

**Background:** Microsatellites or simple sequence repeats (SSRs) are highly informative molecular markers for various biological studies in plants. In spruce (*Picea*) and other conifers, the development of single-copy polymorphic genomic microsatellite markers is quite difficult, owing primarily to the large genome size and predominance of repetitive DNA sequences throughout the genome. We have developed highly informative single-locus genomic microsatellite markers in black spruce (*Picea mariana*) and red spruce (*Picea rubens*) using a simple but efficient method based on a combination of AFLP and microsatellite technologies.

**Principal Findings:** A microsatellite-enriched library was constructed from genomic AFLP DNA fragments of black spruce. Sequencing of the 108 putative SSR-containing clones provided 94 unique sequences with microsatellites. Twenty-two of the designed 34 primer pairs yielded scorable amplicons, with single-locus patterns. Fourteen of these microsatellite markers were characterized in 30 black spruce and 30 red spruce individuals drawn from many populations. The number of alleles at a polymorphic locus ranged from 2 to 18, with a mean of 9.3 in black spruce, and from 3 to 15, with a mean of 6.2 alleles in red spruce. The polymorphic information content or expected heterozygosity ranged from 0.340 to 0.909 (mean = 0.67) in black spruce and from 0.161 to 0.851 (mean = 0.62) in red spruce. Ten SSR markers showing inter-parental polymorphism inherited in a single-locus Mendelian mode, with two cases of distorted segregation. Primer pairs for almost all polymorphic SSR loci resolved microsatellites of comparable size in *Picea glauca*, *P. engelmannii*, *P. sitchensis*, and *P. abies*.

**Significance:** The AFLP-based microsatellite-enriched library appears to be a rapid, cost-effective approach for isolating and developing single-locus informative genomic microsatellite markers in black spruce. The markers developed should be useful in black spruce, red spruce and other *Picea* species for various genetics, genomics, breeding, forensics, conservation studies and applications.

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## Introduction

Microsatellites or simple sequence repeats (SSR) are highly informative genetic markers. Due to their co-dominant inheritance, high polymorphism, reproducibility and transferability across related species, microsatellites have been widely used for various genetics, genomics, breeding, genetic resource conservation, and forensics studies and applications, e.g., [1–8]. Although, recently with the use of next-generation sequencing (NGS) technologies, genome-wide single nucleotide polymorphism

(SNP) and other markers have become available, microsatellite markers are still highly suitable for population and conservation genetics studies and forensics applications, especially for determining neutral population genetic processes and dynamics. In fact, microsatellites were demonstrated to provide more precise information on population genetic structure than SNPs in four different taxa [9]. Also, highly polymorphic co-dominant markers, such as microsatellites, are needed to align genetic maps.

Various methods and strategies have been used to isolate microsatellite-containing sequences in plants and animals, which are primarily based on the development of microsatellite-enriched and non-enriched genomic libraries [10]. Also, availability of a large number of expressed sequence tag (EST) sequences in the database made it possible to identify microsatellite sequences and develop cDNA-based microsatellite markers in agricultural and horticultural plants, e.g., [11–13] and conifer trees [14,15]. The advantages associated with EST-derived microsatellite markers include high probability of such markers being single-locus types and their ability to detect polymorphism directly in expressed genes. However, EST-derived microsatellite markers show lower polymorphism than genomic microsatellites [7]. This is possibly due to lower mutation rates, conservation and slow evolution of gene sequences and selection constraints on them. Therefore, information obtained from EST-derived microsatellites is likely to be different from that obtained from faster-evolving genomic microsatellites. Also, the expressed portion of the genome is quite small, especially in plants with large genome size; thus, cDNA-based microsatellite markers may have limited and non-random representation of the genome. Hence, it is important to develop genomic microsatellites. More recently, massive parallel sequencing of the whole transcriptomes and genomes can reveal a large number of sequences with microsatellite repeats.

Due primarily to huge genome size ( $2\text{--}4 \times 10^{10}$  base pairs) and highly repetitive genomes of conifers [16,17], development of highly informative single-locus genomic microsatellite markers remains a challenge in these long-lived plants. Although a large number of microsatellite-containing sequences can now be identified by whole genome sequencing in conifers [18,19] using NGS platforms, the challenge still remains to isolate and develop genomic microsatellite markers that show simple single-locus DNA variant patterns. Genomic microsatellite markers have been developed for a number of spruce (*Picea*) and other conifer species using non-enriched and microsatellite-enriched [2,20–23] and BAC/YAC genomic libraries [24,25]. However, the number of genomic microsatellites showing single-copy single-locus patterns remains small because a large proportion of genomic microsatellites have shown complex multilocus patterns with a frequent presence of null alleles. The difficulties and challenges have been mainly attributed to the large genome size and the presence of high proportion of redundant repetitive DNA sequences throughout the genomes in conifers [16,17]. In order to minimize the problems, attempts were made to isolate microsatellite markers from unique DNA sequences, using two approaches: low copy enrichment method [26] and under-methylated (UM) DNA method following methylation restriction enzyme (*Mcr*BC) [27]. Of the above two approaches, the low-copy enrichment method was found to be more successful. However, both of these methods are still time consuming and expensive and yielded only a small number of highly informative single-locus microsatellite markers. Development of informative single-locus microsatellite markers was however more successful from cDNA or EST sequences in conifers [13–15].

Here we report a simple and efficient approach for the development of highly informative single-locus genomic microsatellite markers in two widely distributed and economically and ecologically important conifers, black spruce (*Picea mariana*) and red spruce (*Picea rubens*). Microsatellite sequences were identified from microsatellite-enriched AFLP DNA fragments in black spruce. We have determined informativeness of these markers in black spruce and red spruce, their Mendelian single-locus inheritance and linkage in progeny of two controlled crosses of

black spruce, and their cross-species transferability to four other spruce species.

## Materials and Methods

### Plant Material and DNA Extraction

One of the parents (accession # 59) of a three-generation outbred mapping pedigree of black spruce was used for the construction of microsatellite-enriched library. Thirty individuals each of black spruce and red spruce drawn from 12 populations were used to determine the informativeness of the microsatellite markers. Eight individuals each of white spruce (*P. glauca*), Sitka spruce (*P. sitchensis*), Engelmann spruce (*P. engelmannii*), and Norway spruce (*P. abies*) were used for determining the cross-species transferability of the microsatellite markers, with the exception of one marker RPMSA07 for which five individuals of each species were used. The black spruce samples were previously obtained from nine populations located at three sites in Manitoba, Canada [28] as follows: Pine Falls – natural mature (1), post-harvest natural young regeneration (1), and plantation (4); Bissett – natural mature (3), post-harvest natural young regeneration (2), and plantation (3); and Snow Lake – natural mature (3), post-harvest natural young regeneration (6), and plantation (7). The red spruce samples were from three provenances included in a range-wide provenance test established at the Acadia Forest Experiment Station, Fredericton, New Brunswick: Great Smoky Mountain National Park, Tennessee-North Carolina boundary (6), Monongahela National Forest, Pocahontas County, West Virginia (12), and Logan State Forest, Pennsylvania (12). The Norway spruce samples were obtained from the Nova Scotia Department of Natural Resources, Sitka spruce samples from the British Columbia Ministry of Natural Resources, and white spruce and Engelmann spruce samples were from Alberta, Canada. The parents and 110, and 30 progeny of two  $F_2$  controlled crosses of black spruce  $32 \times 40$ , and  $46 \times 14$ , respectively, were used to examine the inheritance and linkage of microsatellite markers. The details on controlled crossing experiments and pedigrees are provided in Kang et al. [6].

Total genomic DNA was extracted from the needle tissues of each individual using Qiagen DNeasy plant mini kit according to the manufacturer's instructions (Qiagen, Toronto, ON, Canada). The quality and quantity of DNA was evaluated using a spectrophotometer and agarose gel electrophoresis.

### Isolation of Microsatellite DNA Sequences and Development of SSR Markers

An AFLP-based microsatellite-enriched library was constructed using total genomic DNA of black spruce accession 59 and applying the magnet beads method [29,30], with minor modifications. Genomic DNA ( $\sim 600$  ng) was digested with two different restriction endonucleases *Eco*RI and *Mse*I at  $37^\circ\text{C}$  for 2 h and ligated with *Eco*RI and *Mse*I adaptors using the AFLP Analysis System I (Invitrogen Life Technologies, Burlington, ON, Canada). An initial PCR reaction was conducted in a total volume of 200  $\mu\text{l}$ , containing 2.5 mM  $\text{MgCl}_2$ , 0.16 mM each of dNTPs, 10 pmoles of *Eco*RI adaptor-specific primer (5'-GACTGCGTACCAATTC-3') and 60 pmoles of *Mse*I adaptor-specific primer (5'-GATGAGTCCTGAGTAA-3'), 10 unit of Taq polymerase (Fermentas, Fisher Scientific, Ottawa, ON, Canada) and 10  $\mu\text{l}$  of purified adaptor-ligated DNA as templates. Amplification was performed with 10 cycles each of denaturation at  $90^\circ\text{C}$  for 30 sec, annealing at  $56^\circ\text{C}$  for 60 sec and extension at  $72^\circ\text{C}$  for 60 sec. The amplification products were hybridized with 100 pmoles of 3'-biotin-labelled (AG)<sub>15</sub> oligonucleotides, and the target DNA

fragments were recovered using Dynabeads M-280 Streptavidin (DynaL Biotech, Life technologies, Burlington, ON, Canada) in a STEX buffer (100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA and 0.1% Triton-100, pH 8.0) for 30 min [29,30]. We used (AG)<sub>15</sub> probe for microsatellite enrichment because AG repeats were found to be most abundant in white spruce [2]. The enriched DNA fragments were amplified again with 25 cycles using the same cycling conditions. The purified PCR products were inserted into the pCR2.1 TOPO vectors and then transformed into One Shot Competent Cells provided in the TA cloning kit (Invitrogen Life Technologies, Burlington, ON, Canada), following the manufacturer's instructions. The bacteria were plated on LB agar plates containing X-gal and 100 µg/ml ampicillin. The positive clones (white and some light blue colonies) were screened by PCR using the Universal M13 Reverse Primer and the T7 Promoter Primers (Invitrogen Life Technologies, Burlington, ON, Canada). Colony blot hybridization was carried out using biotinylated (AG)<sub>15</sub> probe and BrightStar BioDetect non-isotopic detection kit according to the user's manual (Ambion, Life Technologies, Burlington, ON, Canada) to further screen and identify the plasmids containing microsatellite sequences.

The putative microsatellite-containing clones were grown overnight in 3 ml of LB broth with 50 µg/ml ampicillin. Subsequently, plasmids were extracted using a QIAprep Spin Miniprep Kit (Qiagen, Toronto, ON, Canada). The purified DNA fragments were sequenced using the Universal M13 Reverse Primer labelled with IRdye 800 and the T7 Promoter Primer labeled with IRDye 700 with Thermo Sequenase fluorescent-labeled primer cycle sequencing kit (Amersham Pharmacia Biotech, Quebec, Canada).

The sequence data were analyzed using the E-SEQ v2 program and the Align IR program (LICOR, Lincoln, Nebraska, USA). Primers were designed for the non-redundant SSR-containing sequences using Primer 3 program [31]. The oligonucleotide primers complementary to the regions flanking the identified SSR repeat motifs were synthesized incorporating IRDye 700 or 800 labels by the Integrated DNA Technologies (Coralville, IA, USA), and used for amplification by PCR.

### PCR Amplification, Optimization and Visualization of Microsatellites

PCR amplification for all microsatellite markers was performed in a 20 µl reaction volume, with 100 ng of purified spruce genomic DNA, 1.5 mM of MgCl<sub>2</sub>, 200 µM of each of dNTPs, 200 ng of BSA, 1 U of *Taq* DNA polymerase (Fermentas, Fisher Scientific, ON, Canada) and 7.5 pmole of each forward and reverse primers. To optimize the amplification conditions for each microsatellite locus, the following temperature cycling parameters were tested: denaturation for 3 min at 94°C, followed by two cycles of 30 sec each at 94°C, 55–60°C and 72°C; and 38 cycles each of 15 sec at 94°C, 55–60°C and 72°C, with a final extension step of 10 min at 72°C. For the primers yielding no, weak or multi-locus amplification products using the above PCR profiles, a touchdown protocol was used: initial denaturation at 94°C for 3 min followed by two cycles each of 30 sec at 94°C, 60°C and 72°C; followed by 13 cycles each of 94°C for 15 sec, 60 ↓ 54°C for 15 sec (0.5°C per cycle) and 72°C for 15 sec; and 30 cycles each of 15 sec at 94°C, 54°C and 72°C, with a final cycle at 72°C for 10 min.

After amplification, the PCR products were separated on a 6.5% polyacrylamide gel at 1500 volts for 2.5 h on the LICOR sequencing system IR 4200 (LiCor, Lincoln, NE, USA) with IRDye 700 and 800 labelled size standards (IR 50–350 bp). The genotypes of spruce individuals were determined by their allelic

constitution manually. The DNA fragment size of individual alleles was determined based on the size standard used.

### Informativeness, Inheritance and Linkage of Microsatellite Markers

The informativeness of the microsatellite loci was evaluated in black spruce and red spruce by determining the number of alleles and their frequency, observed heterozygosity ( $H_o$ ), and expected heterozygosity ( $H_e$ ). Also polymorphism information content (PIC) for each locus was calculated as follows [32]:  $1 - \sum P_i^2$  where  $P_i$  is the frequency of the  $i$ th allele in 30 individuals of a species examined. The PIC is the same as expected heterozygosity ( $H_e$ ).

The inheritance, segregation patterns and linkage of the polymorphic microsatellite markers were examined in two three-generation outbred pedigrees, which consisted of the parents and  $F_2$  progenies of two controlled crosses (32×40 (110 progeny), and 46×14 (30 progeny) of black spruce. The linkage relationships among the seven microsatellite loci (RPMSA04, RPMSA07, RPMSA09, RPMSA12, RPMSA13, RPMSA17, and RPMSA33), showing interparental polymorphisms in the 32×40 cross have been previously determined [6]. In the 46×14 cross, the two-point linkage was determined among five informative loci (RPMSA04, RPMSA11, RPMSA12, RPMSA26, and RPMS33), and between RPMSA13 and RPMSA22 by investigating joint segregation and independent assortment of their microsatellite DNA variants. Due to certain logistics reasons, three different sets of 30  $F_2$  progeny of the 46×14 cross were used for determining the inheritance and linkage of the microsatellite markers: set 1 (RPMSA04, RPMSA11, RPMSA12, RPMSA26, and RPMS33), set 2 (RPMSA07), and set 3 (RPMSA13, and RPMSA22). Therefore, the pair-wise linkage relationships among all eight markers showing interparental polymorphism in this cross could not be tested. The null hypothesis that the two loci in a pair are independently assorted, was tested by the  $\chi^2$  goodness-of-fit test. Since the expected number of progeny in genotypic classes for five microsatellite pairs (RPMSA04-RPMSA11, RPMSA11-RPMSA12, RPMSA11-RPMSA26, RPMSA11-RPMSA33, and RPMSA13-RPMSA22) was less than the minimum of 5 required for a valid  $\chi^2$  test [33], we pooled adjacent two or four progeny genotypic classes to obtain a joint class with an expected progeny number >5 to perform the  $\chi^2$  test [33]. This resulted in a total of two or four joint progeny classes.

### Cross-species Transferability of Microsatellite Markers

The number of alleles observed at each locus in eight individuals (five individuals for RPMSA07) of Norway spruce, Sitka spruce, white spruce, and Engelmann spruce were determined to test the cross-species transferability of the microsatellite markers developed from black spruce.

## Results

### Construction of Microsatellite-enriched Library and Identification of SSR-containing Sequences

The microsatellite-enriched library was constructed from genomic DNA of black spruce after restriction with a rare and a frequent endonucleases and enrichment with biotinylated (AG)<sub>15</sub> oligonucleotides. One-thousand twenty-nine white and light blue colonies were isolated. Of these, 311 were identified as positive potentially containing DNA inserts after the first colony PCR screening with the T7 Promoter and the Universal M13 reverse primers. The second screening with the (AG)<sub>15</sub> oligonucleotides as probes using non-isotopic detection method resulted in excluding

**Table 1.** Polymorphic microsatellite markers/loci showing simple single-locus patterns; and their repeat motifs, primer sequences, annealing temperatures, and GenBank accession numbers.

| Microsatellite locus | Repeat type  | Primers (5'-3')                                  | Annealing temperature (°C) | GenBank Accession No. |
|----------------------|--|--|----------------------------|-----------------------|
| RPMSA01              | (TA) <sub>9</sub> (GA) <sub>7</sub> (GATA) <sub>13</sub> | GGAGCTAAACACATTTGGTACAGG<br>GAAACCATTGATGTGGGTTG | 60 ↓ 54                    | KJ847201              |
| RPMSA04              | (CT) <sub>23</sub>                                       | GTGAGATTTTGGCAGCAACA<br>TGATCACCTTGCTCAAAGA      | 60                         | KJ847204              |
| RPMSA05              | (GA) <sub>20</sub>                                       | CCCTATCCCCTGAAATCC<br>CTTATGGGCTCCACCACACT       | 60                         | KJ847205              |
| RPMSA06              | (CT) <sub>2</sub> (GA) <sub>6</sub>                      | CTCGACAGACCCCTCTTTTG<br>CAAGTCTCGGTTCTCCTCCA     | 60 ↓ 54                    | KJ847206              |
| RPMSA07              | (CT) <sub>16</sub>                                       | TGAAGAAGCAAGTGGGCTCT<br>TGCATTGATCTCTCCCTTT      | 60 ↓ 54                    | KJ847207              |
| RPMSA09              | (AG) <sub>8</sub>  | CACCTCAGTTCACACCTGCT<br>TTCCTCTCCAAGAATGTGC      | 60                         | KJ847209              |
| RPMSA11              | (GA) <sub>21</sub>                                       | GACCCTAGATTTTGGGGTAT<br>CCCCCTCAGTAATCCAAC       | 60                         | KJ847211              |
| RPMSA12              | (TC) <sub>9</sub> CC(TC) <sub>4</sub>                    | AACGAGGTTTCATCCCATCTG<br>TACGCTCAATGTCGATGAGG    | 60 ↓ 54                    | KJ847212              |
| RPMSA13              | (GA) <sub>9</sub>  | AACCATGAAACCTTAGCGACT<br>TGAGGACTTAGGCCACATT     | 60                         | KJ847213              |
| RPMSA15              | (GA) <sub>7</sub> (AGAAT)(GA) <sub>4</sub>               | ATCGATAGGCTTCAAGAGG<br>TGTGCCCTCATGTGCTATGT      | 60 ↓ 54                    | KJ847215              |
| RPMSA17              | (CT) <sub>12</sub>                                       | CAACGACTGCAACTGGGTACT<br>CAACCATAGACACGCAACCA    | 60                         | KJ847217              |
| RPMSA19              | (CT) <sub>12</sub>                                       | TAGCCAATACAATGCCAAGG<br>ATCAGAGCGAAGTTGGAG       | 60                         | KJ847219              |
| RPMSA22              | (TC) <sub>6</sub> /(TC) <sub>14</sub> /(GA) <sub>4</sub> | GCACGTGCATGTTCTCTGTC<br>TGATGCAGATGAATGAGAG      | 58                         | KJ847222              |
| RPMSA26              | (CT) <sub>2</sub> C(CT) <sub>9</sub>                     | GCTGTAGGGTTGATATTTGC<br>TGTGTGAGAGATAAGTGTGAG    | 65 ↓ 60                    | KJ847226              |
| RPMSA27              | (TC) <sub>22</sub> (TA) <sub>19</sub>                    | ATATTGCAATGAGAGAGCAATC<br>TGTAGGCCCATGATAATGTA   | 55                         | KJ847227              |
| RPMSA33              | (GA) <sub>9</sub>  | ACACACATGAACACATGAGC<br>GCTGTATGGATTCCGTATGA     | 60 ↓ 55                    | KJ847233              |

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203 clones from the putative SSR-containing clones. Sequencing of the remaining 108 putative positive clones revealed 94 unique sequences containing microsatellite repeats with mono- (8.5%), di- (87.3%), tri- (3.2%), and tetranucleotide (1%) motifs (Table S1).

The DNA insert sizes of the 94 microsatellite-containing clones ranged from 148 bp to 1,273 bp, with an average of 462 bp. The majority of clones (77.4%) contained a moderate insert size of 200–600 bp, while five clones (BS-013, 019, 026, 236 and 245) contained larger DNA inserts (>800 bp) and three clones (BS-039, 119 and 241) smaller inserts (<200 bp). Thirty-nine (41.5%) of the identified microsatellites were perfect, 27 (28.7%) imperfect and 28 (29.8%) compound (Table S1). In terms of repeats, GA/CT motif was the most common, accounting for 84.6% of the perfect repeats. One clone (BS-236) contained a simple AT motif and the other two TG/CA motifs. A perfect trinucleotide (CTT) repeat motif was also found in one sequence (BS-120). Among the compound microsatellites, 25 were composed of two repeat types

and three of three repeat types. Twenty-one out of 28 compound microsatellites were interrupted with one or more nucleotides (Table S1). The overall repeat motif number ranged from 4 to 54 units (Table S1).

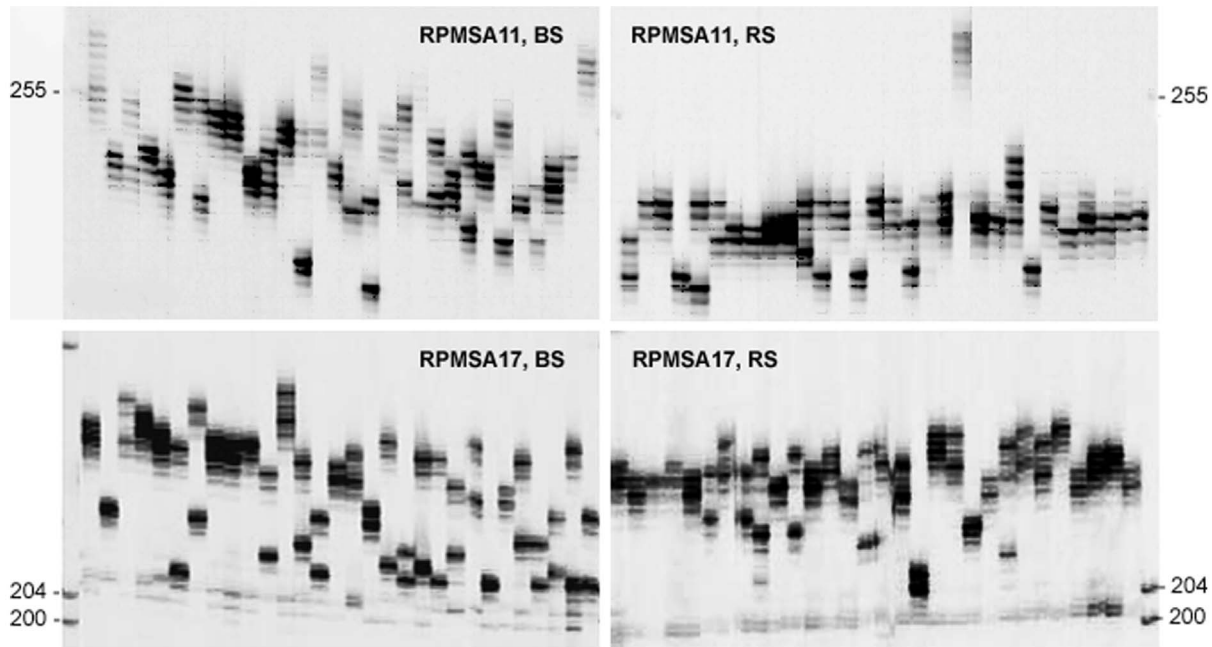
### Microsatellite Markers and Their Informativeness in Black Spruce and Red Spruce

Primer pairs were designed from the flanking regions of microsatellites for selected 34 of the 94 well-characterized microsatellite-containing sequences (Table S2). Each of these primer pairs were initially tested for amplification of microsatellite DNA variants in three black spruce genotypes (59, 63 and N2). A variety of PCR conditions were tested. Of this set, 22 (64.70%) primer pairs yielded single-locus microsatellite variant patterns. The remaining 13 SSR primers either did not yield any amplicon or showed multiple DNA fragment patterns. Among the 22 microsatellite primer pairs resolving microsatellites with simple

**Table 2.** Informativeness of microsatellite markers in black spruce and red spruce.

| Microsatellite locus | Black Spruce      |                   |                |                     | Red Spruce        |                   |                |                     |
|----------------------|-------------------|-------------------|----------------|---------------------|-------------------|-------------------|----------------|---------------------|
|                      | Number of alleles | Allele size range | H <sub>o</sub> | H <sub>e</sub> /PIC | Number of alleles | Allele size range | H <sub>o</sub> | H <sub>e</sub> /PIC |
| RPMSA01              | 1                 | 206               | 0              | 0                   | 1                 | 170               | 0              | 0                   |
| RPMSA04              | 9                 | 105–148           | 0.133          | 0.796               | 3                 | 124–146           | 0.172          | 0.161               |
| RPMSA05              | 17                | 165–231           | 0.267          | 0.909               | Multilocus        |                   |                |                     |
| RPMSA06              | 1                 | 208               | 0              | 0                   | 3                 | 206–210           | 0.172          | 0.587               |
| RPMSA07              | 12                | 141–181           | 0.433          | 0.902               | 6                 | 153–161           | 0.214          | 0.692               |
| RPMSA09              | 4                 | 164–170           | 0.400          | 0.576               | 6                 | 146–200           | 0.217          | 0.617               |
| RPMSA11              | 16                | 227–267           | 0.900          | 0.908               | 10                | 227–267           | 0.467          | 0.779               |
| RPMSA12              | 3                 | 185–202           | 0.100          | 0.661               | 5                 | 184–206           | 0.267          | 0.639               |
| RPMSA13              | 15                | 182–210           | 0.690          | 0.868               | 13                | 178–210           | 0.733          | 0.851               |
| RPMSA15              | 7                 | 192–203           | 0.900          | 0.745               | 4                 | 197–209           | 0.900          | 0.663               |
| RPMSA17              | 14                | 204–234           | 0.867          | 0.894               | 10                | 206–226           | 0.667          | 0.818               |
| RPMSA19              | 8                 | 137–151           | 0.467          | 0.57                | 4                 | 139–145           | 0.167          | 0.541               |
| RPMSA22              | 8                 | 220–238           | 0.167          | 0.747               | 5                 | 202–232           | 0.133          | 0.769               |
| RPMSA26              | 2                 | 119–121           | 0.067          | 0.34                | 3                 | 119–123           | 0.200          | 0.636               |
| RPMSA27              | 18                | 168–222           | 0.367          | 0.908               | 15                | 154–216           | 0.800          | 0.847               |
| RPMSA33              | 15                | 176–216           | 0.667          | 0.868               | 5                 | 192–208           | 0.600          | 0.662               |
| <b>Mean</b>          | <b>9.38</b>       |                   | <b>0.401</b>   | <b>0.669</b>        | <b>6.20</b>       |                   | <b>0.381</b>   | <b>0.618</b>        |

H<sub>o</sub>, observed heterozygosity; H<sub>e</sub>, expected heterozygosity; PIC, polymorphic information contents.  
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**Figure 1. Allelic variation at two microsatellite loci.** Allelic variation among black spruce (BS) and red spruce (RS) individuals at RPMSA11 and RPMSA17 microsatellite loci.  
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single-locus patterns, 16 primer pairs produced consistent and clear scorable amplicons in 30 black spruce individuals and 15 primer pairs in 30 red spruce individuals (Table 1; Table 2; Fig. 1). The optimal annealing temperatures for each of the 16 primer pairs are listed in Table 1.

In black spruce individuals, two loci, RPMSA01 and RPMSA06, both compound microsatellites (Table 1) were found to be monomorphic (Table 2). The numbers of alleles detected at the polymorphic markers were quite variable ranging from 2 (RPMSA26) to 18 (RPMSA27), with an average of 9.3 alleles per locus (Table 2). The DNA fragment sizes of alleles ranged from 105 bp to 267 bp (Table 2). Among the red spruce individuals, the RPMSA01 locus was monomorphic, while the RPMSA05 primers produced multilocus patterns. The number of alleles ranged from 3 to 15 at a locus in red spruce (Table 2), with a mean of 6.2 alleles per locus. The most informative locus in red spruce in terms of the number of alleles was RPMSA27, with 15 alleles detected in the 30 individuals. The frequencies of individual alleles at the polymorphic loci in 30 individuals of black spruce are provided in Table S3, whereas that for red spruce in Table S4. Most of the alleles had low frequencies as expected for highly polymorphic loci. In black spruce, the allele frequencies at a polymorphic locus ranged from 0.017 at 13 loci to 0.80 at RPMSA26 (Table S3). In red spruce, frequencies of individual alleles ranged from 0.017 at seven polymorphic loci to 0.588 at RPMSA09 (Table S4). Red spruce individuals had 30 alleles not detected in black spruce samples, whereas black spruce samples had 70 alleles not detected in red spruce samples at 15 loci (RPMSA05 was excluded because its primers produced multilocus patterns in red spruce and genotypes of individual samples could not be scored) (Table S3; Table S4). The expected heterozygosity or PIC values for the polymorphic microsatellite loci varied from 0.34 (RPMSA26) to 0.909 (RPMSA05), with an average of 0.67 in black spruce, and from 0.161 (RPMSA04) to 0.851 (RPMSA13), with an average of 0.62

in red spruce (Table 2). The observed heterozygosity values for the polymorphic microsatellite loci varied from 0.100 (RPMSA12) to 0.900 (RPMSA11 and RPMSA15), with an average of 0.401 in black spruce, and from 0.133 (RPMSA22) to 0.900 (RPMSA15), with an average of 0.381 in red spruce (Table 2).

#### Inheritance and Linkage of SSRs in Black Spruce

Ten microsatellite loci were found to be polymorphic between the parents of one or both controlled crosses (Table 3; Fig. 2). At each of the 10 loci, the progenies of each cross segregated into two to four genotypic classes as expected for single-locus Mendelian inheritance patterns (Table 3). However, significant deviations from the expected Mendelian progeny ratios were observed in two cases. Segregation of alleles at RPMSA04 in one of the two controlled crosses (46×14) conformed to the expected Mendelian ratios. However, the observed ratio in the progenies of the other cross (32×40) departed from the Mendelian expectations as evaluated by  $\chi^2$ -tests,  $P \leq 0.05$  (Table 3). The second case was with RPMSA17 with four segregating alleles (Table 3).

In the 32×40 pedigree, seven microsatellite loci showed independent assortment, and six of them were previously mapped on to six linkage groups (LG) [6]: LG01: RPMSA13; LG02: RPMSA09; LG04: RPMSA12; LG05: RPMSA33; LG06: RPMSA17; LG 07: RPMSA4. In the 46×14 cross, the Chi-square goodness-of-fit tests indicated that the observed numbers of genotypes were in agreement with those expected for independent assortment of two loci in each of the 11 two-locus combinations tested (Table S5).

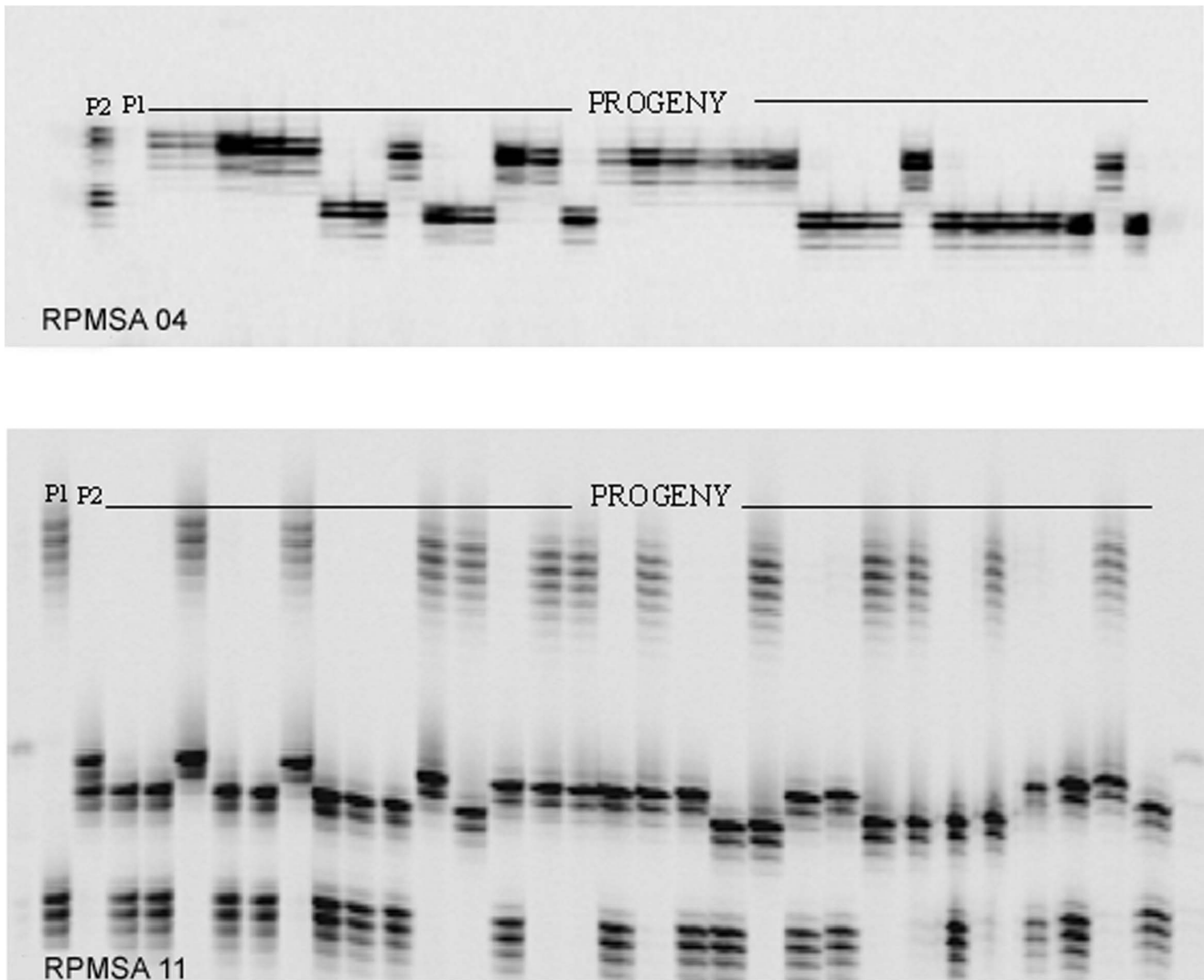
#### Cross-species Transferability of Black Spruce Microsatellite Markers in other *Picea* Species

Reproducible amplifications of the microsatellites of most of the 15 loci were obtained in Norway spruce, Sitka spruce, Engelmann

**Table 3.** Inheritance of microsatellite markers in  $F_2$  progenies of two black spruce controlled crosses.

| Primer/Locus | Cross   | Parental genotypes | Number of progeny | Progeny genotypes (number)    | Expected ratio | $\chi^2$ | P     |
|--------------|---------|--------------------|-------------------|-------------------------------|----------------|----------|-------|
| RPMSA04      | 32 × 40 | BO × AB            | 110               | AO(18);BB(BO(54);AB(38)       | 1:2:1          | 7.31     | 0.026 |
|              | 46 × 14 | OO × AB            | 30                | AO(16);BO(14)                 | 1:1            | 0.13     | 0.715 |
| RPMSA07      | 32 × 40 | AO × BC            | 110               | AB(33);AC(27);BO(26);CO(24)   | 1:1:1:1        | 1.64     | 0.651 |
|              | 46 × 14 | AO × AB            | 30                | AA(7);AO(8);AB(7);BO(8)       | 1:1:1:1        | 0.53     | 0.912 |
| RPMSA09      | 32 × 40 | AB × AA            | 110               | AB(61);AA(49)                 | 1:1            | 1.31     | 0.253 |
| RPMSA11      | 46 × 14 | BC × AD            | 29                | AB(7);BD(7);AC(5);CD(10)      | 1:1:1:1        | 1.76     | 0.624 |
| RPMSA12      | 32 × 40 | AB × AA            | 110               | AB(49);AA(61)                 | 1:1            | 1.31     | 0.253 |
|              | 46 × 14 | OO × AO            | 30                | AO(14);OO(16)                 | 1:1            | 0.13     | 0.715 |
| RPMSA13      | 32 × 40 | AB × CD            | 108               | AD(31);AC(26);BC(20);BD(31)   | 1:1:1:1        | 3.04     | 0.386 |
|              | 46 × 14 | BC × AC            | 30                | AB(8);AC(9);BC(8);CC(5)       | 1:1:1:1        | 1.20     | 0.753 |
| RPMSA17      | 32 × 40 | AD × BC            | 92                | AC (27);AB(10);BD (36);CD(19) | 1:1:1:1        | 16.09    | 0.001 |
| RPMSA22      | 46 × 14 | BB × AB            | 30                | AB(13);BB(17)                 | 1:1            | 0.03     | 0.853 |
| RPMSA26      | 46 × 14 | BB × AB            | 30                | AB(14);BB(16)                 | 1:1            | 0.53     | 0.467 |
| RPMSA33      | 32 × 40 | BC × AC            | 104               | AC(29);AB(30);BC(17);CC(28)   | 1:1:1:1        | 4.23     | 0.238 |
|              | 46 × 14 | AB × BB            | 30                | AB(13);BB(17)                 | 1:1            | 0.53     | 0.467 |

Note: Only those microsatellite loci that showed interparental polymorphisms and segregation in the progeny are included. The alleles are coded alphabetically for simplicity to follow their inheritance and segregation. O = Null allele.  
doi:10.1371/journal.pone.0103789.t003



**Figure 2. Inheritance of microsatellite DNA variants in black spruce.** Inheritance and segregation pattern of microsatellite markers at (a) RPMSA04, and (b) RPMSA11 in  $F_2$  progeny of  $46 \times 14$  controlled cross. The genotypes of the parents and progeny are provided in Table 3. doi:10.1371/journal.pone.0103789.g002

spruce, and white spruce (Table 4). The fragments amplified from the four species were in the size range as observed in black spruce.

Among the 15 primers tested, nine yielded polymorphic amplicons in at least two species (Table 4). The RPMSA33 primers yielded polymorphic PCR products in Engelmann spruce and white spruce, a monomorphic product in Sitka spruce and no product in Norway spruce. The RPMSA05 primers detected several loci in Norway spruce, Sitka spruce, Engelmann spruce, white spruce and red spruce. The RPMSA01 SSR locus was monomorphic in all spruce species tested, including black spruce and red spruce. Interestingly, RPMSA06, RPMSA9, and RPMSA19 resolved polymorphic microsatellite DNA variants in black spruce and/or red spruce, but these loci were monomorphic in the individuals of other four species. While two or three alleles were detected at RPMSA26 in black spruce and red spruce samples (Table 2), the primers for this locus did not show any amplification in the four species.

## Discussion

### Isolation and Development of Microsatellite DNA Markers

We have developed highly informative single-locus genomic microsatellite DNA markers in black spruce and red spruce using an AFLP-based microsatellite enrichment and isolation method. This is the first report for microsatellite marker development in red spruce. A few genomic microsatellite markers have recently been reported for black spruce [34]. We demonstrate that 14 of the 16 microsatellite markers characterized are informative in black spruce and red spruce. Of these, seven (RPMSA05, RPMSA07, RPMSA11, RPMSA13, RPMSA17, RPMSA27, and RPMSA33) were the most informative loci in black spruce, each with over 10 alleles and expected heterozygosity/PIC close to 0.90. In red spruce, four loci (RPMSA11, RPMSA13, RPMSA17, and RPMSA27), with 10 or more alleles at a locus were the most informative. The levels of allelic polymorphisms of the microsatellite loci were positively correlated with the repeat numbers present in the original clones isolated from black spruce ( $r = 0.484$ ,



**Table 4.** Transferability of microsatellite markers to other four spruce species: number of alleles detected in selected individuals of each species.

| Locus    | Number of alleles |              |                  |              |
|----------|-------------------|--------------|------------------|--------------|
|          | Norway spruce     | Sitka spruce | Engelmann spruce | White spruce |
| RPMSA01  | 1                 | 1            | 1                | 1            |
| RPMSA04  | 2                 | 8            | 3                | 1            |
| RPMSA05  | Multilocus        | Multilocus   | Multilocus       | Multilocus   |
| RPMSA06  | 1                 | 1            | 1                | 1            |
| RPMSA07* | 6                 | 5            | 4                | 5            |
| RPMSA09  | 1                 | 1            | 1                | 1            |
| RPMSA11  | 3                 | 10           | 10               | 9            |
| RPMSA12  | 5                 | 2            | 2                | 3            |
| RPMSA13  | 6                 | 9            | 9                | 7            |
| RPMSA17  | 10                | 7            | 8                | 6            |
| RPMSA19  | 1                 | 1            | 1                | 1            |
| RPMSA22  | 7                 | 5            | 7                | 4            |
| RPMSA26  | -                 | -            | -                | -            |
| RPMSA27  | 6                 | 6            | 6                | 8            |
| RPMSA33  | -                 | 1            | 5                | 4            |

Note:

\*Five individuals each of *P. abies*, *P. sitchensis*, *P. glauca* and *P. engelmannii* were used to amplify the SSR locus RPMSA07, whereas eight individuals per species were used to amplify other 14 SSR loci.

- SSR locus not detected.

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$p = 0.04$ ) and red spruce ( $r = 0.480$ ,  $p = 0.04$ ). These results are consistent with the positive correlation observed between the repeat length and the polymorphism in white spruce [2], Norway spruce [22], and other plants [35,36]. In contrast, no such positive correlation between the microsatellite repeat length and resultant polymorphism was observed in some plant species [37,38]. Microsatellite loci with compound repeats are generally believed to show lower polymorphism than loci with simple repeats. While this was true for two (RPMSA01, and RPMSA06) of the six microsatellite loci with compound repeats in our study, the locus RPMSA27 with compound CT and AT repeats showed the highest polymorphism in black spruce and red spruce (Table 2).

Several approaches have been used to isolate and develop microsatellite DNA markers from spruce species (Table 5). The comparison with other approaches (Table 5) suggests that the AFLP-based approach that we have reported here, provided the most efficient method for isolating and developing highly informative single-locus microsatellites with simple patterns in spruce. The success rate was over 250% higher than that was observed for microsatellite marker development in black spruce from standard microsatellite-enriched genomic libraries in our own lab (Table 5; Joy and Rajora, unpublished). It is worth noting that the success rate was even higher than that observed for isolating and developing microsatellites from cDNA and EST sequences [15] or from low-copy sequences selected from microsatellite-enriched genomic libraries [39] in spruce. We used *EcoRI* as one of the two restriction enzymes, which is methylation sensitive. The repetitive elements in the genome are highly methylated [40]. Therefore, methylated sites in the genome may have been preferentially excluded by using *EcoRI* and this may have enriched single- or low copy sequences in the microsatellite-enriched AFLP fragments library. Similar AFLP-based approach

has also been successfully used to develop microsatellite markers in pear (*Pyrus pyrifolia*) [29] and peach (*Prunus persica*) [30].

An AFLP-based method named Fast Isolation by AFLP of Sequences Containing Repeats (FIASCO) was recently introduced [10], which was believed to be a simple, rapid and effective method for microsatellite isolation from genomic DNA *de-novo*. However, this method did not prove effective in conifers as the percentage of microsatellite-containing clones was relatively low (between 29.5 to 43.1%) [23,41,42]. The FIASCO method does not apply a microsatellite enrichment step, whereas our method employs microsatellite enrichment followed by double screening for microsatellite-enriched clones. In our study, the enriched genomic library was screened with a colony PCR followed by a hybridization with (AG)<sub>15</sub> oligonucleotides as probes. This most likely helped us to exclude the majority of non-microsatellite containing colonies and increased the efficiency of isolating microsatellite-containing clones. Of the 108 clones sequenced, 97 clones contained microsatellites and only nine (8.3%) did not. The sequences of two clones were of poor quality.

High allelic polymorphism and  $H_e$ /PIC and simple single-locus inheritance patterns of the microsatellites developed in this study suggest that the developed markers could be used for various genetics, genomics, breeding, DNA fingerprinting, tree forensic and genetic resource conservation studies and applications in black spruce and red spruce. Indeed six (RPMSA04, RPMSA09, RPMSA12, RPMSA13, RPMSA17, and RPMSA33) of the 14 polymorphic microsatellite markers developed have already been mapped to six linkage groups in black spruce [6]. Thus, our study provides a rich resource of highly informative single-copy microsatellite DNA markers in spruce.

**Table 5.** Comparison of development efficiency of single-locus or simple pattern microsatellite markers in *Picea*.

| Species                                       | Sources  | Clones screened | Positive clones | Clones sequenced | Unique sequences | SSR containing sequences | Primer pairs designed (P) | SSR loci with simple patterns (S) | Rate of loci with simple pattern (%) (P/S) | Reference                  |
|---|--|-----------------|-----------------|------------------|------------------|--------------------------|---------------------------|-----------------------------------|--|----------------------------|
| <i>Picea mariana</i>                          | Enriched genomic DNA AFLP library  | 1029            | 108             | 108              | 103              | 94                       | 34                        | 22                                | 64.70                                      | This study                 |
| <i>Picea mariana</i>                          | Enriched genomic DNA library   | 2819            | 1612            | 120              | 90               | 73                       | 40                        | 10                                | 25.00                                      | Roy & Rajora (unpublished) |
| <i>Picea mariana</i>                          | Enriched genomic library   | >200            | >200            | 20               |                  |                          |                           | 7                                 |  | Dobrzyniecka et al. [34]   |
| <i>Picea glauca</i>                           | Non-enriched & enriched genomic library                                    | 5404            | 91              | 32               |                  | 23                       | 16                        | 8                                 | 50.0                                       | Rajora et al. [2]          |
| <i>Picea glauca</i>                           | Enriched genomic library   | 200             |                 | 60               |                  | 56                       | 34                        | 13                                | 38.23                                      | Hodgetts et al. [21]       |
| <i>Picea glauca</i> & <i>Picea sitchensis</i> | cDNA library EST sequences   |                 |                 |                  | 34,846           | 188                      | 44                        | 25                                | 56.81                                      | Rungis et al. [15]         |
| <i>Picea abies</i>                            | Enriched genomic library - trinucleotide repeats                           |                 | 121             | 100              | 100              | 85                       | 55                        | 23                                | 41.82                                      | Scotti et al. [22]         |
| <i>Picea abies</i>                            | Enriched genomic library – dot blot selection of low copy number sequences | 600             | 150             | 150              |                  | 108                      | 53                        | 33                                | 62.26                                      | Scotti et al. [39]         |
| <i>Picea abies</i>                            | Genomic library  | ~65,000         | 223             | 46               | 46               |                          | 36                        | 7                                 | 19.44                                      | Pfeiffer et al. [20]       |
| <i>Picea abies</i>                            | cDNA library   | 50,000          | 23              | 23               | 23               | 16                       | 16                        | 6                                 | 37.50                                      | Scotti et al. [61]         |

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## Microsatellite Diversity and Divergence in Black Spruce and Red Spruce

Black spruce individuals had 150 alleles at 16 microsatellite loci and 133 alleles at 15 loci after excluding RPMSA05 that showed multilocus patterns in red spruce (Table 2). Red spruce individuals had 93 alleles at the same 15 SSR loci. Also, the expected and observed heterozygosity values were lower in red spruce individuals. This is consistent with previous reports based on allozyme and cDNA sequence-tagged sites (STS) markers that red spruce harbours relatively lower genetic diversity [43–45] as compared to sympatric black spruce and white spruce. Genetic diversity at the studied microsatellite loci could be regarded as high in black spruce and moderate in red spruce accessions studied. However, although for a limited number of samples, the microsatellite allelic variability results suggest that red spruce cannot be considered as genetically depauperate as reported by Perron et al. [44].

Red spruce individuals had a number of microsatellite alleles (30) that were not detected in black spruce individuals (Table 2; Table S3; Table S4). Also locus RPMSA06 was monomorphic for one allele in black spruce but had three alleles in red spruce individuals. Based on the STS markers, Perron et al. (2000) concluded that genetic diversity of red spruce is a subset of black spruce genetic diversity [44]. Our microsatellite results, albeit for a small but equal number of samples for black and red spruce, do not support this conclusion. The microsatellite locus RPMSA01 was monomorphic with species-specific alleles in black spruce (allele 206) and red spruce (allele 170). If these results hold true for a larger number of black spruce and red spruce individuals, the RPMSA01 locus could be used to unambiguously differentiate between closely related black and red spruce. These two spruce species hybridize naturally where their ranges overlap, and are difficult to differentiate morphologically. Also, no species-specific allozyme, ribosomal, mitochondrial or chloroplast DNA markers could be found for these species [46–48]. However, RAPD markers specific to black and red spruce have been reported [49]. But RAPDs are not reliable markers until converted to codominant and reliable SCAR markers. Therefore, codominant markers, such as the microsatellite DNA markers, could provide excellent tools to differentiate between black spruce and red spruce.

## Inheritance, Segregation and Linkage of Microsatellite Markers

The Mendelian inheritance and segregation of microsatellite variants in the progenies of two controlled crosses (Table 3) demonstrate that the variants at each of the 10 microsatellite loci are controlled by a single nuclear locus and that the microsatellite variants (alleles) at these loci are codominantly inherited in black spruce. Segregation distortions were observed at two microsatellite loci: RPMSA04, and RPMSA17 (Table 3). However, for RPMSA04, segregation distortion was observed in progeny of one controlled cross and not in that of the other. Segregation distortion or transmission ratio distortion of molecular markers has been reported in many plant species, including spruce [6,50,51]. In a black spruce-red spruce  $BC_1$  backcross, 47% of SAMPL (selectively amplified microsatellite polymorphic loci) markers showed distorted segregation ratios [50]. In Norway spruce, three (2.3%) out of the 133 tests with 51 SSR markers showed a significant deviation from Mendelian segregation ratios [51]. In other species, for example *Isis*, approximately 1/3 of all microsatellite markers in each linkage map revealed significant transmission ratio distortion [52]. In our study, two of 15 (13.3%) tests significantly departed from the Mendelian ratios. This level

was comparable and falls within the range of distortion values observed in intraspecific crosses [53].

Segregation/transmission distortion is a common phenomenon in plants [54], with backcrosses and  $F_2$  showing high percentage of loci showing transmission ratio distortion [54–56]. Several biological mechanisms and experimental errors in scoring the mapping population genotypes can cause deviations from expected segregation ratios. Although we thoroughly verified marker scoring, one of the two parents in one of the two cases showing segregation distortions had null alleles. This may have caused some ambiguity in scoring the progeny genotypes. The presence of null alleles can cause segregation distortion in the crosses [57]. Biological mechanisms that can cause segregation distortion include chromosome/chromatin loss [54], divergence of the parental genotypes [53,54], inbreeding depression, presence of the transmission distorter loci or genomic regions [55], meiotic drive locus [56], and genetic load and recessive lethal alleles [58]. We examined the inheritance of microsatellite markers in  $F_2$  progeny of black spruce. The parents of this progeny were related by descent. Black spruce shows high inbreeding depression [59]. Therefore, the observed segregation distortion may have been caused by inbreeding depression.

Our study in combination with the results reported by Kang et al. [6] suggests that there is no linkage among RPMSA04, RPMSA07, RPMSA09, RPMSA11, RPMSA12, RPMSA13, RPMSA17, RPMSA26, and RPMSA33, and between RPMSA13 and RPMSA22. Therefore, these microsatellite loci could be simultaneously used in population genetic studies in black spruce.

## Cross-species Transferability of Microsatellite Markers

Primers for 14 out of 16 microsatellite loci developed in black spruce were able to amplify microsatellites in at least four other species, with primers for 12 loci yielding amplification products in all six spruce species. In red spruce, primers for all 16 microsatellite loci worked, resolving single-locus variants for 15 loci and multilocus patterns for one microsatellite locus (Table 2). Thus, it could be inferred that most of the microsatellite markers developed from black spruce could potentially be used for various genetics, genomics and breeding studies in five other spruce species tested. The transferability rate in this study was higher than previously reported for spruce [2,15]. It has been suggested that if a particular microsatellite locus could be resolved in black, white, red, and Sitka spruce by using the same primers, it is likely that the primers will be widely transferable to other spruce species as well [15]. The high cross-species transferability observed in the present study indicates that the regions flanking the microsatellite sequences are well conserved in the six spruce species studied. The cross species transferability of microsatellite markers in *Picea* species will reduce the cost of marker development.

The success of cross-species transferability of microsatellite loci depends on the evolutionary relatedness of the taxa being examined. In this study, primers developed for all 16 microsatellite loci from black spruce sequences resolved microsatellites in red spruce. This is consistent with close genetic and phylogenetic relationships between these two species [44,60]. It is worth noting that primers for RPMSA05 resolved a single highly polymorphic microsatellite locus in black spruce but yielded multilocus patterns in red spruce, and other four spruce species tested.

## Conclusions

We have developed a reliable and highly efficient approach for isolating and developing highly informative microsatellite DNA markers with simple single-locus patterns in black spruce and red

spruce. The markers inherited in a Mendelian single-locus codominant fashion and were not linked. Most of the microsatellite markers could be successfully transferred to Norway, Sitka, white, and Engelmann spruce. The new set of microsatellite markers developed here could be used for various genetics, genomics, breeding, DNA fingerprinting, tree forensics and genetic resource conservation studies and applications in six spruce and potentially other spruce species. The microsatellite markers reported here are based on developing primers for only 34 out of 94 unique SSR-containing sequences. Thus, additional markers could be developed from 60 microsatellite-containing sequences that we did not use.

## Supporting Information

### Table S1 Microsatellite-containing clones, repeat motifs and insert size in black spruce.

(DOCX)

### Table S2 Microsatellite markers screened; their repeat motifs and primer sequences.

(DOCX)

### Table S3 Allele frequencies at characterized microsatellite loci in 30 individuals of black spruce (*Picea mariana*).

(DOCX)

### Table S4 Allele frequencies at characterized microsatellite loci in 30 individuals of red spruce (*Picea rubens*).

(DOCX)

### Table S5 Joint two-locus segregation patterns and chi-square analyses for testing of linkage between micro-

### satellite DNA loci in the 46×14 cross of black spruce (*Picea mariana*).

(DOCX)

### Table S6 Microsatellite-containing sequences of black spruce used for primer design.

(DOCX)

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## Author Contributions

Conceived and designed the experiments: YS OPR. Performed the experiments: YS NF. Analyzed the data: YS NF OPR. Contributed reagents/materials/analysis tools: OPR. Wrote the paper: OPR YS NF. Contributed to the conception of idea for developing microsatellite markers from microsatellite-enriched AFLP fragments, developed and optimized the protocol, developed and optimized microsatellite markers and contributed to the manuscript writing and revision: YS. Contributed to the lab work and data scoring and analysis for determining the informativeness, inheritance and cross-species transferability of the microsatellite markers, and to the manuscript preparation: NF. Principal Investigator of the project and contributed to the study conception, experimental design, data analysis, overall research directions and guidance, and manuscript writing and revision, and obtained funding and provided laboratory facilities: OPR.

## References

- Rajora OP, Rahman MH, Buchert GP, Dancik BP (2000) Microsatellite DNA analysis of genetic effects of harvesting in old-growth eastern white pine (*Pinus strobus*) in Ontario. *Mol Ecol* 9: 339–348.
- Rajora OP, Rahman MH, Dayanandan S, Mosseler A (2001) Isolation, characterization, inheritance and linkage of microsatellite DNA markers in white spruce (*Picea glauca*) and their usefulness in other spruce species. *Mol Gen Genet* 264: 871–882.
- Rahman MH, Rajora OP (2001) Microsatellite DNA analysis of somaclonal variation in tissue culture micropropagated trembling aspen plants. *Plant Cell Rep* 20: 531–536.
- Rahman MH, Rajora OP (2002). Microsatellite DNA fingerprinting, differentiation and genetic relationships of clones, cultivars and varieties of six poplar species from three sections of the genus *Populus*. *Genome* 45: 1083–1094.
- Rajora OP, Rahman MH (2003) Microsatellite DNA and RAPD fingerprinting, identification and genetic relationships of hybrid poplar (*Populus xcanadensis*) cultivars. *Theor Appl Genet* 106: 470–477.
- Kang BY, Mann IK, Major JE, Rajora OP (2010) Near-saturated and complete genetic linkage map of black spruce (*Picea mariana*). *BMC Genomics* 11:515.
- Fageria MS, Rajora OP (2013) Effects of harvesting of increasing intensities on genetic diversity and population structure of white spruce. *Evolutionary Applications* 6:778–794.
- Bashalkhanov S, Eckert AJ, Rajora OP (2013) Genetic signatures of natural selection in response to air pollution in red spruce (*Picea rubens*, Pinaceae). *Mol Ecol* 22: 5877–5889.
- Hamblin MT, Warburton ML, Buckler ES (2007) Empirical comparison of simple sequence repeats and single nucleotide polymorphisms in assessment of maize diversity and relatedness. *PLoS One* 2: e1367.
- Zane L, Bargelloni L, Patarnello T (2002) Strategies for microsatellite isolation: a review. *Mol Ecol* 11:1–16.
- Cho YG, Ishii T, Temnykh S, Chen X, Lipovich L, et al. (2000) Diversity of microsatellites derived from genomic libraries and GenBank sequences in rice (*Oryza sativa* L.). *Theor Appl Genet* 100:713–722.
- Thiel T, Michalek W, Varshney RK, Graner A (2003) Exploiting EST databases for the development and characterization of gene-derived SSR-markers in barley (*Hordeum vulgare* L.). *Theor Appl Genet* 106: 411–422.
- Scott KD, Egger P, Seaton G, Rossetto M, Ablett EM, et al. (2000) Analysis of SSRs derived from grape ESTs. *Theor Appl Genet* 100:723–726.
- Liewlaksaneeyanawin C, Ritland CE, El-Kassaby YA, Ritland K (2004) Single-copy, species-transferable microsatellite markers developed from loblolly pine ESTs. *Theor Appl Genet* 109: 361–369.
- Rungis D, Berube Y, Zhang J, Ralph S, Ritland CE, et al. (2004) Robust simple sequence repeat markers for spruce (*Picea* spp.) from expressed sequence tags. *Theor Appl Genet* 109: 1283–1294.
- Murray BG (1998) Nuclear DNA amounts in gymnosperms. *Ann Bot* 82 (suppl 1): 3–15.
- Kinlaw CS, Neale DB (1997) Complex gene families in pine genomes. *Trends Plant Sci* 2: 356–359.
- Nystedt B, Street NR, Wetterbom A, Zuccolo A, Lin YC, et al. (2013) The Norway spruce genome sequence and conifer genome evolution. *Nature* 497(7451):579–584.
- Biról I, Raymond A, Jackman SD, Pleasance S, Coope R, et al. (2013) Assembling the 20 Gb white spruce (*Picea glauca*) genome from whole-genome shotgun sequencing data. *Bioinformatics* 29:1492–1497.
- Pfeiffer A, Olivieri AM, Morgante M (1997) Identification and characterization of microsatellites in Norway spruce (*Picea abies* K.). *Genome* 40: 411–419.
- Hodgetts RB, Aleksuk MA, Brown A, Clarke C, Macdonald E, et al. (2001) Development of microsatellite markers for white spruce (*Picea glauca*) and related species. *Theor Appl Genet* 102: 1252–1258.
- Scotti I, Magni F, Paglia GP, Morgante M (2002) Trinucleotide microsatellites in Norway spruce (*Picea abies*): their features and the development of molecular markers. *Theor Appl Genet* 106: 40–50.
- Li Y, Liang L, Ge XJ (2010) Development of microsatellite loci for *Pinus koraiensis* (Pinaceae). *Am J Bot* 97: e39–e41.
- Georgi LL, Wang Y, Yvergnaux D, Ormsbee T, Inigo M, et al. (2002) Construction of a BAC library and its application to the identification of simple sequence repeats in peach [*Prunus persica* (L.) Batsch]. *Theor Appl Genet* 105: 1151–1158.
- Chen H, Pulido JC, Duyk GM (1995) MATS: a rapid and efficient method for the development of microsatellite markers from YACs. *Genomics* 25: 1–8.
- Elsik CG, Williams CG (2001) Low-copy microsatellite recovery from a conifer genome. *Theor Appl Genet* 103: 1189–1195.
- Zhou Y, Bui T, Auckland LD, Williams CG (2002) Undermethylated DNA as a source of microsatellites from a conifer genome. *Genome* 45: 91–99.
- Rajora OP, Pluhar SA (2003) Genetic diversity impacts of forest fires, forest harvesting and alternative reforestation practices in black spruce (*Picea mariana*). *Theor Appl Genet* 106: 1203–1212.

29. Yamamoto T, Kimura T, Shoda M, Ban Y, Hayashi T, et al. (2002) Development of microsatellite markers in the Japanese pear (*Pyrus pyrifolia* Nakai). *Molecular Ecology Notes* 2: 14–16.
30. Yamamoto T, Mochida K, Imai T, Shi YZ, Ogiwara I, et al. (2002) Microsatellite markers in peach [*Prunus persica* (L.) Batsch] derived from an enriched genomic and cDNA libraries. *Molecular Ecology Notes* 2: 298–301.
31. Rozen S, Skaletsky H (2000) Primer 3 on the WWW for general users and for biologist programmers. In: Clifton NJ, editor. *Methods in Molecular Biology*. Humana Press, Totowa, NJ: pp 365–386.
32. Cuc LM, Mace ES, Crouch JH, Quang VD, Long TD, et al. (2008) Isolation and characterization of novel microsatellite markers and their application for diversity assessment in cultivated groundnut (*Arachis hypogaea*). *BMC Plant Biol* 8: 55.
33. Sokal RR, Rohlf FJ (1995) *Biometry*. Third edition. W.H. Freeman and Company, New York.
34. Dobrzeznicka S, Nkongolo KK, Michael P, Wyss S, Mehes M (2009) Identification and characterization of microsatellite markers for genetic analysis of black spruce (*Picea mariana* (Mill.) populations. *Silvae Genetica* 58:168–172.
35. Taramino G, Tarchini R, Ferrario S, Lee M, Pe' ME (1997) Characterization and mapping of simple sequence repeats (SSRs) in *Sorghum bicolor*. *Theor Appl Genet* 95: 66–72.
36. Areshchenkova T, Ganai MW (2002) Comparative analysis of polymorphism and chromosomal location of tomato microsatellite markers isolated from different sources. *Theor Appl Genet* 104: 229–235.
37. Donini P, Stephenson P, Bryan GJ, Koebner RMD (1998) The potential of microsatellites for high throughput genetic diversity assessment in wheat and barley. *Genetic Resources and Crop Evolution* 45: 415–421.
38. He GH, Meng RH, Newman M, Gao GQ, Pittman RN, et al. (2003) Microsatellites as DNA markers in cultivated peanut (*Arachis hypogaea* L.) *BMC Plant Biol* 3:3.
39. Scotti I, Paglia GP, Magni F, Morgante M (2002) Efficient development of dinucleotide microsatellite markers in Norway spruce (*Picea abies* Karst.) through dot-blot selection. *Theor Appl Genet* 104: 1035–1041.
40. Rabinowicz PD, Citek R, Budiman MA, Andrew Nunberg A, Bedell JA, et al. (2005) Differential methylation of genes and repeats in land plants. *Genome Res* 2005 15: 1431–1440.
41. Yang JB, Li HT, Li DZ, Liu J, Gao LM (2009) Isolation and characterization of microsatellite markers in the endangered species *Taxus wallichiana* using the FIASCO method. *HortScience* 44:2043–2045.
42. Miao YC, Lang XD, Li SF, Su JR, Wang YH (2012) Characterization of 15 polymorphic microsatellite loci for *Cephalotaxus oliveri* (Cephalotaxaceae), a conifer of medicinal importance. *Int J Mol Sci* 13:11165–11172.
43. Hawley GJ, DeHayes DH (1994) Genetic diversity and population structure of red spruce (*Picea rubens*). *Can J Bot* 72: 1778–1786.
44. Perron M, Perry DJ, Andalo C, Bousquet J (2000) Evidence from sequence-tagged-site markers of a recent progenitor-derivative species pair in conifers. *Proc Natl Acad Sci USA* 97: 11331–11336.
45. Rajora OP, Mossler A, Major JE (2000) Indicators of population viability in red spruce, *Picea rubens*. II. Genetic diversity, population structure, and mating behaviour. *Can J Bot* 78: 941–956.
46. Eckert RT (1989) Genetic variation in red spruce and its relation to forest decline in northeastern United States. In: Bucher JB, Bucher-Wallin I (eds) *Air pollution and forest decline*. IUFRO, Birmensdorf, pp 319–324.
47. Bobola MS, Eckert RT, Klein AS (1992) Restriction fragment variation in the nuclear ribosomal DNA repeat unit within and between *Picea rubens* and *Picea mariana*. *Can J For Res* 22: 255–263.
48. Bobola MS, Eckert RT, Klein AS, Strapelfeldt K, Smith DE, et al. (1996) Using nuclear, and organelle DNA markers to discriminate among *Picea rubens*, *Picea mariana*, and their hybrids. *Can J For Res* 26: 433–443.
49. Perron M, Gordon AG, Bousquet J (1995) Species-specific RAPD fingerprints for the closely related *Picea mariana* and *Picea rubens*. *Theor Appl Genet* 91: 142–149.
50. Yazdani R, Scotti I, Jansson G, Plomion C, Mathur G (2003) Inheritance and diversity of simple sequence repeat (SSR) microsatellite markers in various families of *Picea abies*. *Hereditas* 138: 219–227.
51. Kang BY, Major JE, Rajora OP (2011) A high-density genetic linkage map of a black spruce (*Picea mariana*) × red spruce (*Picea rubens*) interspecific hybrid. *Genome* 54:128–143.
52. Tang SX, Okashah RA, Knapp SJ, Arnold ML, Martin NH (2010) Transmission ratio distortion results in asymmetric introgression in Louisiana *Iris*. *BMC Plant Biol* 10:48.
53. Jenczewski E, Gherardi M, Bonnin I, Proserpi JM, Olivieri I, et al. (1997) Insight on segregation distortions in two intraspecific crosses between annual species of *Medicago* (Leguminosae). *Theor Appl Genet* 94: 682–691.
54. Zamir D, Tadmor Y (1986) Unequal segregation of nuclear genes in plants. *Bot Gaz* 147: 355–358.
55. Fishman L, Aagaard J, Tuthill JC (2008) Towards the evolutionary genomics of gametophytic divergence: patterns of transmission ratio distortion in monkey flower (*Mimulus*) hybrids reveal a complex genetic basis for conspecific pollen precedence. *Evolution* 62: 2958–2970.
56. Fishman L, Willis JH (2005) A novel meiotic drive locus almost completely distorts segregation in *Mimulus* (monkeyflower) hybrids. *Genetics* 169: 347–353.
57. Reece KS, Ribeiro WL, Gaffney PM, Carnegie RB, Allen SK Jr (2004) Microsatellite marker development and analysis in the eastern oyster (*Crassostrea virginica*): confirmation of null alleles and non-Mendelian segregation ratios. *J Hered* 95: 346–352.
58. Bradshaw HD Jr, Stettler RF (1994) Molecular genetics of growth and development in *Populus*. II. Segregation distortion due to genetic load. *Theor Appl Genet* 89: 551–558.
59. Morgenstern EK (1972) Preliminary estimates of inbreeding in natural populations of black spruce, *Picea mariana*. *Can J Genetics Cytol* 14:443–446.
60. Ran JH, Wei XX, Wang XQ (2006) Molecular phylogeny and biogeography of *Picea* (Pinaceae): Implications for phylogeographical studies using cytoplasmic haplotypes. *Mol Phylogenet Evol* 41:405–419.
61. Scotti I, Magni F, Fink R, Powell W, Binelli G, et al. (2000) Microsatellite repeats are not randomly distributed within Norway spruce (*Picea abies* K.) expressed sequences. *Genome* 43: 41–46.