Real-Time PCR and analysis of amplicon melting curves to assess the suitability of SSR loci of Scots pine for multiplexing

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Abstract. Nuclear microsatellite markers are considered to be among the most powerful tools for assessing genetic resources. To date, a great variety of primers for SSR loci of pines has been developed. As regards Scots pine, selection of neutral steadily amplifiable loci not containing null alleles and their multiplexing are high-priority tasks, as there are no generally accepted multiplex panels of loci for this species. Authors of published multiplexes tend not to use loci due to their unstable amplification or the presence of single nucleotide repeats. The aim of the paper was to create the new multiplexes of reliable previously published pine nSSR loci in view of preliminary data based on the analysis of melting curves of products after Real-Time polymerase chain reaction (PCR) for each locus separately. Initially, microsatellite multiplexes were created using Multiplex Manager 1.0. DNA was extracted by the CTAB method from samples of pine needles. Real-Time PCR was performed in CFX96 thermal cyclers (BIO-RAD, USA). Fragment analysis of PCR products labeled with FAM, HEX and ROX dyes was performed on a 3500 genetic analyzer (Applied Biosystems). GeneScanTM 500 LIZ size standard was used. As a result of the study, 3 multiplexes have been proposed.

1. Introduction
Nuclear microsatellite markers (SSR markers) offer convenient, reliable and relatively inexpensive means that can be used for the wide range of practical applications in forestry, such as assessment of genetic diversity in natural woody plant populations, identification of clones and elite trees (or plus trees), and proof of origin of parent tree descendants.

SSR markers were proposed in 1989 [1]. Since the late 1990s, various authors have developed a large number of primers for SSR loci of different pine species. Given the complexity of development of SSR markers, researchers tried to apply the proposed primers to other species of the genus Pinus and even on other species of the family Pinaceae. The use of primers developed for other species is the most applicable for EST-SSR markers characterized by lower variability [2].

The proposed SSR loci were originally developed from enriched genomic libraries (G) [3], low-copy libraries (LC) [4], undermethylated DNA libraries (UM) [5], cDNA libraries (EST) [6-8] and chloroplast genomes [9, 10].

In the 1990s and 2000s the development of nuclear SSR markers was very labor-intensive due to lack of data on a whole genome sequencing of pine species and the need to confirm that the inheritance of the proposed loci was codominant.

The published loci were multiplexed in different combinations by researchers focusing on the genetic diversity and structure of pine species populations [11-13].
In addition to the difficulties arising from the presence of null alleles when using SSR markers, the results were inaccurate due to unstable amplification and the presence of single nucleotide repeats in loci with dinucleotide motifs.

Following the research work, the authors rejected a number of loci or whole multiplexes, or excluded them from data analysis [14, 15]. We reviewed the literature and selected the loci not rejected by other authors [16].

The aim of the paper was to compile the new multiplexes of reliable previously published pine nSSR loci in view of preliminary data based on the analysis of melting curves of products after Real-Time polymerase chain reaction (PCR) for each locus separately.

2. Experimental Part

2.1. Materials and Methods

The characteristics of the used loci are shown in Table 1.

| Microsatellite locus       | Motifa  | Fragment size (bp) | Tₐ (°C)b | SSR origin | Number of alleles | Reference |
|----------------------------|---------|--------------------|----------|------------|-------------------|-----------|
| lw_isotig07383             | (GAT)8  | 191                | 55       | EST        | 3                 | [6]       |
| lw_isotig21953             | (ATGGG)7| 208                | 55       | EST        | 7                 | [6]       |
| lw_isotig26230             | (TA)10  | 260                | 55       | EST        | 3                 | [6]       |
| lw_isotig00080             | (CCG)6  | 177                | 55       | EST        | 3                 | [6]       |
| lw_isotig01420             | (CTG)5  | 174                | 50       | EST        | 3                 | [6]       |
| lw_isotig05123             | (GAG)6  | 166                | 55       | EST        | 2                 | [6]       |
| lw_isotig11166             | (TA)7   | 140-160            | 55       | EST        | 5                 | [6]       |
| Ctg4363                    | (AT)20  | 80-105             | 52       | UM         | 8                 | [2]       |
| PtTx4001                   | (GT)15  | 160-187            | 52       | UM         | 8                 | [5]       |
| psy117                     | (TA)7   | 219–251            | 55       | EST        | 6                 | [8]       |
| Psy157                     | (ACC)7  | 187–202            | 55       | EST        | 6                 | [8]       |
| PtTx3020                   | A16(CAA)9| 200-230           | 52       | LC         | 6                 | [4]       |

a Motif is a repeated nucleotide sequence.
b Tₐ (°C) is a primer annealing temperature.

DNA was isolated from Scots pine needles dried in silica gel using the CTAB (cetyl trimethyl ammonium bromid) method [17]. The purity and concentration of the extracted DNA were determined spectrophotometrically using an ultra-flat spectrometer SPECTROstar Nano (BMG Labtech GmbH, Germany). We used 18 DNA samples from pines of the Porkhov district population (the Pskov Region, Northwest Russia) in Real-Time experiments. PCR was performed in PCR thermocyclers CFX96 (BIO-RAD, USA). Each amplification involved the use of controls without a DNA template. The reaction volume was 20 μl. The PCR mixture was composed of: 1x ready-mix for PCR qPCRmix-HS (Evrogen, Russia); 0.1 μmol forward and reverse primers, 1x EvaGreen (Biotium, 20x aqueous solution), and 15 ng of DNA.

The range of annealing temperature for each primer pair was specified using amplification protocol with a temperature gradient from 53.6 °C to 63.6 °C and an analysis of the melting curves (relative fluorescence units (RFU) and RFU peaks (d(RFU)/dT)) of amplification products after Real-Time PCR.
To make new multiplex panels, the free program Multiplex Manager version 1.0 [18] was used. Multiplex PCR was performed with the use of forward primers labeled with fluorescent dyes FAM, HEX, ROX, and reverse unlabeled primers under the following conditions: 5 min at 95 °C followed by 35 cycles: 30 s at 95 °C, 45 s at 58 °C, 30 s at 72 °C, and final elongation for 10 min at 72 °C; melting curve from 65 °C to 95 °C in 0.5 °C increments for 5 s. Graphical data was analyzed using Bio-Rad CFX Manager 3.1 (USA).

Microsatellite analysis was performed on a genetic analyzer 3500 (Applied Biosystems) for 31 trees from Udmurt republic, Novgorod region, Perm Territory and Vologda Region. The graphs of capillary electrophoresis were processed using the GeneScan™ 500 LIZ size standard with the GeneMapper 5.0 software (Applied Biosystems) to determine the lengths of the obtained fragments.

2.2. Results and Discussion
According to its developers, the Multiplex Manager 1.0 addresses and optimizes several factors: the range of allele sizes, annealing temperatures, primer complementarity, heterozygosity, number of alleles, and genetic linkage [18]. However, we were unable to use the multiplexes created by the software without any modifications due to the lack of amplification of a part of the loci in multiplex PCR. It might be related to the possible grouping loci originating from the same DNA region, as well as with the great similarity of a number of primers for different loci as identified by the software.

Analysis of melting curves is now widely used to confirm the specificity of amplification reactions and other applications: detecting SNPs, identifying species of organisms and studying DNA methylation profiles [19].

To confirm the opportunity of amplification of different loci in one tube and to specify the range of acceptable annealing temperatures, PCR was performed with a temperature gradient for each pair of primers separately.

In addition to estimating the range of temperatures at which amplification occurs, the same experiments provided the data on the specificity of the reaction products at different annealing temperatures.

Comparison of the melting peaks of amplification products in these experiments revealed that the specificity of amplification of most loci was stable over wide temperature ranges (table 2, figure 1).

Table 2. Amplification of selected loci in the experiments with temperature gradient in the thermal cycler CFX96 (BIO-RAD, USA).

| Ta (°C) | lw_isotig21953 | lw_isotig20230 | lw_isotig00880 | lw_isotig051122 | psyl17 | Cg4363 | Psyl57 | lw_isotig07383 | lw_isotig11166 | lw_isotig01420 | PtTX4001 |
|--------|----------------|----------------|----------------|-----------------|--------|--------|--------|----------------|----------------|----------------|----------|
| 63.6   | ▲ ▲ ▲ ▲ ▲ ▲ ▲ | 63.6           |
| 63.1   | ▲ ▲ ▲ ▲ ▲ ▲ ▲ | 63.1           |
| 61.9   | ▲ ▲ ▲ ▲ ▲ ▲ ▲ | 61.9           |
| 60.0   | ▲ ▲ ▲ ▲ ▲ ▲ ▲ | ▲ ▲ ▲ ▲ ▲ ▲ ▲ ▲ ▲ | ▲ ▲ ▲ ▲ ▲ ▲ ▲ ▲ | ▲ ▲ ▲ ▲ ▲ ▲ ▲ |
| 57.5   | ▲ ▲ ▲ ▲ ▲ ▲ ▲ | ▲ ▲ ▲ ▲ ▲ ▲ ▲ ▲ ▲ | ▲ ▲ ▲ ▲ ▲ ▲ ▲ |
| 55.6   | ▲ ▲ ▲ ▲ ▲ ▲ ▲ | ▲ ▲ ▲ ▲ ▲ ▲ ▲ ▲ ▲ | ▲ ▲ ▲ ▲ ▲ ▲ ▲ |
| 54.3   | ▲ ▲ ▲ ▲ ▲ ▲ ▲ | ▲ ▲ ▲ ▲ ▲ ▲ ▲ ▲ ▲ | ▲ ▲ ▲ ▲ ▲ ▲ ▲ |
| 53.6   | ▲ ▲ ▲ ▲ ▲ ▲ ▲ | ▲ ▲ ▲ ▲ ▲ ▲ ▲ ▲ ▲ | ▲ ▲ ▲ ▲ ▲ ▲ ▲ |

a ▲ – stable amplification of all DNA samples.
b △ – some DNA samples are not amplified.
Figure 1. Melting peaks of PCR products of loci (a) – Ctg4363; (b) – lw_isotig01420 in the experiment with a gradient temperature from 53.6 to 63.6 °C. On the X-axis – the melting point of amplicons (°C); on the Y-axis – d(RFU)/dT (RFU – the relative fluorescence units).

Figure 2. Melting peaks of PCR products in locus PtTx3020 in case of amplification at annealing temperature of: (a) – 54.3 °C; (b) – 57.5 °C; (c) – 60.0 °C; (d) – 61.9 °C. On the X-axis - melting point of amplicons (°C); on the Y-axis - d(RFU)/dT (RFU – the relative fluorescence units).

For some loci, the experiment showed that different products were synthesized at differ annealing temperatures. Apparently, in the case of melting shoulder peaks of DNA fragments (figure 2 (b)), a
mixture of products is produced in PCR. Figure 2 shows the melting peaks of the amplicons of locus PtTX3020 synthesized at annealing temperatures of 54.3, 57.5, 60.0 and 61.9 °C. As can be seen in figure 2, at least 3 fragments were produced with melting peaks at temperatures of 62.0, 74.0 and 77.5 °C. In PCR at an annealing temperature of 60 °C, two major amplicons were produced, one of which was apparently, a dimer of primers.

In contrast, loci lw_isotig21953, lw_isotig26230, lw_isotig00080, lw_isotig05123, psy117, cgt4363, psy157, lw_isotig07383, lw_isotig11166, lw_isotig01420 и PtTX4001, are characterized by the presence of a narrow melting peak of products in a wide range of annealing temperatures which points to the specificity of amplification at these temperatures. This presents great opportunities to combine different loci into multiplexes.

As you can see in figure 2, PtTx3020 locus is not very convenient for multiplexing. The developers of primers for this locus indicated a very high annealing temperature, 65 °C [4]. According to the experiment with the temperature gradient (figure 2), given that more specific products were synthesized at higher annealing temperatures, this locus could be amplified at annealing temperatures from 60 to 62 °C. At higher annealing temperatures were observed products with the same melting peaks with significantly fewer d(RFU)/dT. In our laboratory, this locus was poorly amplified in all tested multiplexes at different annealing temperatures when the applicable reagents and equipment were used.

Although the Real-Time PCR makes it possible to get information on the specificity of products in individual loci quickly as compared with electrophoresis in polyacrylamide gels, and determine threshold cycles of amplification (Cq), the results obtained with this method should be considered as preliminary. The multiplexes were tested upon completion of multiplex PCR with labeled forward primers using fragment analysis on a 3500 genetic analyzer (Applied Biosystems). This instrument has a higher sensitivity and takes into account the fluorescence of specific dyes labelling the amplicons.

As a result of this work, we proposed three multiplexes of nuclear microsatellite loci listed in table 3.

| Multiplex | Locus                  | Dye | Fragment size (bp) | Ta (°C) |
|-----------|------------------------|-----|--------------------|---------|
| I         | lw_isotig21953         | HEX | 190-280            | 58.0    |
|           | lw_isotig26230         | ROX | 254-258            |         |
|           | lw_isotig05123         | ROX | 166-172            |         |
|           | Psy117                 | FAM | 219-251            |         |
|           | lw_isotig00080         | FAM | 177-183            |         |
| II        | Ctg4363                | FAM | 94-116             | 58.0    |
|           | Psy157                 | HEX | 187-202            |         |
|           | lw_isotig07383         | ROX | 191-209            |         |
| III       | lw_isotig11166         | HEX | 140-160            | 58.0    |
|           | lw_isotig01420         | ROX | 177-189            |         |
|           | PtTX 4001              | HEX | 195-231            |         |

The results of the proposed multiplexes testing are given in figure 3.
Figure 3. Results of fragment analysis of Scots pine multiplexes of nSSR loci on a 3500 genetic analyzer: (a) – multiplex I (loci lw_isotig21953, lw_isotig26230, lw_isotig05123, Psyl17, lw_isotig00080); (b) – multiplex II (loci Ctg4363, Psyl57, lw_isotig07383); (c) – multiplex III (loci lw_isotig11166, lw_isotig01420, PtTX4001). GeneScan™ 500 LIZ Size Standard. X-axis is DNA fragment length (bp); Y-axis is relative fluorescence units (RFU).

3. Conclusion
According to the data received, to obtain working multiplex panels of microsatellites suitable for assessing the biological diversity of populations and identifying valuable pine genotypes, it would be highly beneficial to use Real-Time PCR with intercalating dyes for double-stranded DNA (EvaGreen, SybrGreen I) with analysis of melting curves of products for each locus.

In experiments with Real-Time PCR, the annealing temperature ranges were specified for each locus separately, at which the synthesis of a specific product takes place.

We proposed three multiplexes that were successfully tested on 31 trees of pine from Udmurt republic, Novgorod region, Perm Territory, Vologda Region, in multiplex PCR with the detection of results by fragment analysis on a 3500 genetic analyzer (Applied Biosystems).
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