

ring in Llao Llao population, but we cannot generalize such observation to all of the populations of the species.

Indirect methods, like HW test, suffer from the shortcoming of being based on too many assumptions, which can hardly be controlled in order to test them separately. Direct procedures of testing any kind of genetic processes should always be preferred. Otherwise, conclusions could be no more than speculation based on mathematical models not well adjusted to the real biological processes.

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PCR-Multiplex of Six Chloroplast Microsatellites for Population Studies and Genetic Typing in *Pinus sylvestris*

By A. DZIALUK and J. BURCZYK

Department of Genetics, Institute of Biology and Environmental Protection, Bydgoszcz University, 85-064 Bydgoszcz, Poland
e-mail: dzialuk@ab-byd.edu.pl; burczyk@ab-byd.edu.pl

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Abstract

The major drawback of microsatellites analysis is that they are expensive to develop, labor-intensive and demand skilled personnel. However, such studies might be still simplified and accelerated by multiplexing of the markers and the use of high-throughput systems for genotyping DNA fragments. In this paper we present a single, simple and highly effective PCR-multiplex reaction composed of six chloroplast microsatellites widely used for population studies in pines but here applied to *Pinus sylvestris*. The reaction allows for rapid genotyping of large sample sizes.

Key words: chloroplast microsatellites; PCR-multiplex, *Pinus sylvestris*; polymorphism; haplotypes.

Introduction

The set of microsatellites for *Pinus* species has been developed by VENDRAMIN et al. (1996) and PROVAN et al. (1998). Since then, the chloroplast microsatellites have become an efficient and powerful marker for studying genetic diversity and differentiation among conifers (ECHT et al., 1998; GUGERLI et al., 2001; WALTER and EPPERSON, 2001). They also have proved to be useful for measuring pollen contamination in seed orchards (PLOMION et al., 2001), or for the identification of the origin of populations with uncertain history (RIBEIRO et al., 2002).

The availability of high-throughput systems for genotyping DNA fragments (automated sequencers) involving multicolor

fluorescent fragment labeling allows for simultaneous analyses of several markers increasing the efficiency of genotyping of large samples, which are typical in population genetic studies. Multiplex PCRs (simultaneous amplification of two or more loci in a single PCR reaction) are common in microbial diagnostics, animal breeding and recently become popular among agricultural crops (TANG et al., 2003, and references therein). Nevertheless, PCR multiplexes were seldom reported for conifers (BOUSQUET and PERRY, 2000; MARSHALL et al., 2002; STOEHR and NEWTON, 2002). Although some authors report the post-PCR multiplexing (pooling) of two or more individually amplified PCR products, and use of the aliquots in a single electrophoresis run (VENDRAMIN et al., 1996; ECHT et al., 1998; WALTER and EPPERSON, 2001), the PCR-multiplexing offers an advantage of reducing time and costs of analyses (NEFF et al., 2000; TANG et al., 2003). The aim of this study was to develop simple (i.e., standardized composition of the PCR reaction as well as standard thermal cycle profile) and highly effective PCR reaction for rapid genotyping of large sample sizes.

Twenty nine individuals representing different clones of Scots pine (*Pinus sylvestris* L.) were sampled from a seed orchard located in Gniewkowo, Poland. Genomic DNA was isolated from 100 mg of frozen needles and additional, for ten randomly chosen trees, from diploid embryos of germinated seed (seedlings 5–10 mm in length) and from haploid megagametophytes. Three different methods of isolation were used: DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions, DELLAPORTA et al. (1983) and DOYLE and DOYLE (1990) protocols. Concentration of DNA samples was measured using DNA calculator (BioPhotometr, Eppendorf).

Multiplex PCRs were performed in 10 µl reaction mixture using a thermocycler (PTC-200, MJ Research). The microsatellites selected to combine in a single multiplex were: Pt15169, Pt26081, Pt30204, Pt36480, Pt71936 (VENDRAMIN et al., 1996), and PCP87314 (PROVAN et al., 1998). The forward primer of each microsatellite was labeled with a specific fluorescent dye (Applied Biosystems), as indicated in Table 1. The final optimal reaction mixture, derived based on the results of several experiments where different concentrations of components were tested, contained 20 ng of template DNA, 1x PCR buffer (10 mM TRIS-HCl, 50 mM KCl; Sigma), 3 mM of MgCl₂, 0.2 mM of each dNTP, 0.04–0.15 µM each of forward and reverse primers (see below and Table 1), 0.4 µg/µl of BSA, and 0.25 U of REDTaq Genomic DNA Polymerase (Sigma). The PCR conditions were as follows: after initial denaturation at 94°C for 5 min, PCR was performed for 30 cycles, each consisting of 94°C for 30 s, 50°C for 1 min and 72°C for 1 min. Final extension was at 72°C for 10 min. PCR products (1 µl) were mixed with 9 µl of FSS (formamided size standard – HD 400 ROX Size Standard (Applied Biosystems): formamide, in ratio 1:24). Microsatellite fragments

were analyzed on ABI Prism 310 DNA Sequencer (Applied Biosystems). The actual sizes of fragments were determined using GeneScan and Genotyper software packages (Applied Biosystems) using internal standards. For simplicity, in this paper the term “locus” will refer to a cpSSR site, while “allele” will refer to a size variant of a cpSSR site (ECHT et al., 1998). Since the chloroplast genome does not recombine, a combination of alleles across loci can be considered a haplotype.

The optimal conditions for multiplex PCR were determined also by altering primer concentration. Primers were initially evaluated by using equimolar concentrations of all primer pairs (150 nM) in individual PCR and analyzed for product size and yields. Next, the multiplex-PCR product yields were used to adjust the concentration of the primer pairs, to give comparable results within the reaction. Concentrations were altered for one primer pair at a time, increased when intensity of the corresponding product was too low, and decreased when intensity of the product was too high. After few primer optimization steps, the final concentration has been determined, that gives comparable yields from each individual primer pair (about 1000 relative fluorescence intensity units). Using the primer ratios determined by sequential optimization, there was no need for further optimizations (e.g. change the other PCR conditions).

Different strategies of DNA extraction resulting in different template qualities may play a crucial role in the efficiency of PCR reaction. We found the same results using different methods of DNA extraction, but it should be noted, that without BSA, the reaction for some individual trees (especially using Qiagen's kit) did not work at all. The best results were obtained when using above mentioned adjuvant at higher concentration than previously reported (e.g. 0.2 mg/µl in PALME et al., 2004). Such high BSA concentration guarantees successful multiplex PCR amplification, apart from template quality.

The optimized PCR multiplex worked well for the various tissues sampled (needles, seed megagametophytes and embryos). The multiplex PCR reaction was successfully used even for single megagametophytes, while some authors reported problems using templates prepared from this type of tissue (BOUSQUET and PERRY, 2000).

Among 29 individuals of Scots pine, the number of alleles per locus ranged from 2 to 5. There were 24 unique haplotypes, however the smaller number of haplotypes than the number of sampled clones (29) may be the result of ramet mislabeling during the seed orchard establishment, which was suspected after our previous isozyme study of that orchard (BURCZYK et al., 2000). An alternative explanation is just that different clones are sharing the same chloroplast haplotypes, either because of shared paternal ancestry or because of lack of marker resolution. More detailed verification of all orchard ramets seems to be necessary in the future.

Because fragment sizes of some of the studied loci do not overlap, a subset of loci included in our multiplex can be used even in non-multicolor labeling DNA fragment analyzers. On the other hand, because the multiplex reaction is highly efficient, multicolor fluorescent labeling may allow to include some additional loci. Nevertheless, the selection of additional loci needs to be careful. Despite that the cpSSR markers proposed by PROVAN et al. (1998) have different primer pairs than those developed by VENDRAMIN et al. (1996), they actually amplify the same microsatellite regions. The primer code denotes the position of the 5' base of sense forward primer in the published *P. thunbergii* cp DNA sequence (VENDRAMIN et al., 1996) and therefore e.g. PCP30277 = Pt30204; PCP71987 = Pt71936. In

Table 1. – Characterization of six multiplexed chloroplast microsatellites used in a PCR-multiplex.

Locus	Fluorescent dye	Primer concentrations	Expected product size	Observed product size	Number of alleles
PCP87314	HEX	40 nM	114–118 ^b	111–114	4
Pt 26081	FAM	50 nM	109–112 ^a	104–105	2
Pt 15169	NED	75 nM	124–128 ^a	122–126	5
Pt 30204	NED	100 nM	141–146 ^a	140–144	5
Pt 36480	FAM	150 nM	143–144 ^a	140–141	2
Pt 71936	HEX	75 nM	141–148 ^a	143–146	4

^a from ROBLEDO et al. (2004)

^b from PROVAN et al. (1998)

our study, the replacement of Vendramin's primer pair Pt87268 by Provan's PCP87314, enabled us to multiplex a set of six chloroplast microsatellites. Such strategy may be also useful to design multiplex reactions even for non-multicolor sequencers.

The high number of chloroplast haplotypes typical in pines (PLOMION et al., 2001; ROBLEDO et al., 2004; this study), despite population genetic studies, can also be used in genetic typing surveys, including forestry management practices or even forensics (WHITE et al., 2000). For example, if the cpSSR profiles of a number of elite mother trees used for commercial seed collections are known, the assay of a sample of seed megagametophytes may verify the origin of the seeds. Also, clonal identification of ramets in clonal seed orchards may be achieved with a greater reliability. This type of investigation will certainly be simplified by the aid of the presented multiplex. We expect that the multiplex optimized in this paper for *Pinus sylvestris* can be effectively used in other pine species.

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