Identification of seeds of *Pinus* species by microsatellite markers

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Abstract: The 276 pair-primers (nuclear and chloroplast microsatellite) developed from seven species of Pinaceae were selected and identified for cross-species transferability to ten *Pinus* species (*P. massoniana, P. kesiya, P. tabulaeformis, P. densiflora, P. thunbergii, P. caribaea, P. taeda, P. yunnanensis, P. densata, P. sylvestris)* belonging to Sect. *Pinus* by BSA (bulked segregate analysis) method. The results showed that 23 of 276 (8.0%) markers were successful to have amplification product in ten species, and 5 of 23 (21.7%) were polymorphic cross species and lack of polymorphic within species. Eight of 10 *Pinus* species were identified by using single primer, two and more combination of primers, but there were still no effective SSR primers for identifying other 2 species (*P. kesiya* and *P. densata*).

Keywords: Identification; Simple sequence repeats (SSRs); *Pinus* species; Microsatellite Markers
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Introduction

Microsatellites, or simple sequence repeats (SSRs), are composed of short tandemly repeated, and two to five nucleotide DNA sequences. The markers are abundant, highly polymorphic and easy to score. Microsatellites are usually characterized by numerous alleles that differ in size by the length of the repeat unit (Tautz 1989; Weber *et al.* 1989) and may be amplified by Polymerase Chain Reaction (PCR) from small amounts of DNA by using specific primers developed from specific species. SSR marker can be useful for genome mapping and genetic fingerprinting, and for identifying seeds and seedlings of closely related species. Previously, several molecular markers, such as Random Amplified polymorphic DNAs (RAPD), Amplified Fragment Length Polymorphism (AFLP) were used widely to identify species, but they have lethal shortcomings. RAPD is usually unstable and the amplification product is not effective marker until it is transferred to Sequence Characterized Amplified Region (SCARs) or Sequence Tag Site (STS) marker. The operating procedures of AFLP are complex and dangerous because of radioactive isotope. Comparatively, SSR marker is developed as a most reliable and simple molecular method to identify seeds of closely-related species.

There are about 38 species and 12 varieties of *Pinus* genera spreading throughout China. Most of the species of Sect. *Pinus* are major commercial trees including *P. sylvestris, P. tabulaeformis, P. densata, P. yunnanensis, P. yunnanensis var. pygnaea, P. kesiya, P. massoniana, P. taiwanensis, P. nigra, P. thunbergii, P. taeda, P. elliottii* and *P. caribaea*. It is difficult to distinguish those seeds clearly according to the morphology, due to the fact that the morphology of seeds and seedling of intra-species is very similar. However, because SSRs are stable and specific loci for species, it is suitable for use to identify seeds and seedlings of *Pinus* species with the molecular level.

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Methods and materials

Sources of DNA samples

Ten species were chosen from Sect. *Pinus* (subgenus *Pinus*) taxa, including *P. massoniana* (10 provenances: 5 from Guangxi, 2 from Zhejiang, 2 from Guizhou and 1 from Fujian), *P. kesiya* (2 provenances from Yunnan), *P. tabulaeformis* (2 provenances from Shanxi), *P. densiflora* and (1 provenance from Shandong), *P. thunbergii* (1 provenance from Shandong), *P. caribaea* and *P. taeda* (2 provenances imported from USA.), *P. yunnanensis* (2 provenaces from Yannan), *P. densata* (2 provenances from Sicuan) and *P. sylvestris* (1 provenance from northeast of china). DNA was extracted from germinants by using 2% CTAB procedure (100 mM Tris-HCl, pH 8.0; 1.4M NaCl; 20mM EDTA; 2% CTAB (W/V); 1% PVP (W/V) and 0.4% β-Mercaptoethanol), (Dellaporta *et al.* 1983).

Microsatellite primer transfer and optimization

The 262 nuclear pair-primers and 14 chloroplast pair-primers were examined for transfer individually, which developed from *Picea abies* (Antomella *et al.* 1997; Scotti *et al.* 2002), *Picea glauca* (Hodgetts *et al.* 2001), *Pinus taeda* (Elsik *et al.* 2001; Zhou *et al.* 2002), *Pinus strobus* (Echt *et al.* 1996), *Pinus pinaster* and *Pinus halepensis* (Gonzalez *et al.* 2002), and *Pinus contorta* var. *latifolia* (Mark *et al.* 1998). PCR optimization was attempted by testing a range of $MgCl_2$, dNTPs and amplification procedure (especially annealing temperature). The 10-μL amplication reaction included 20–50ng of DNA template and final concentrations of 200 mM each dNTP, 2.5mM Mg^{2+}, 500 nM each primer, 0.25U Taq polymerase (TaKaRa, Taq^{TM}) and 1×Buffer (Tris-Cl, 10mM; KCl, 50 mM). A touchdown amplification procedure was run on GeneAmp 9700 thermocycle (Perkin Elmer) by using the following programs: initial pre-denaturation at 94 °C for 5 min, 16 cycles including a denaturing step at 95 °C for 30 s, an annealing step at 58 °C for 30 s (which subsequently was decreased by 0.5 °C every cycle until the final temperature reached 50 °C) and an extension step at 70 °C for 30 s, the next 24 cycles consisting of 95 °C for 30s, 50 °C for 30 s, 70 °C for 30 s, and final extension at 70 °C for 5 min. Amplification products were examined with vertical denaturing polyacrylamide gels. The composition of long gels (1 mm×15 cm) was acrylamide of 8%, 8-M

urea, and 1×TBE. For glass plate preparation, Acrylease™ (Stratagene Cloning systems) was used for a nonstick coating on one plate and bind silane for the gel binding treatment on the other. Gels were electrophoresed at room temperature, with 250 v for 1 h. Separated markers were stained with silver. After electrophoresis, the gel, bound to a glass plate, was fixed for 10 min with 10% alcohol and 0.5% acetic acid, then given two 2-min rinses with d.i. water. The gel was stained for 10 min in 0.15% silver nitrate with gentle swaying and then rinsed 2 times with d.i. water, for 5 min each time. For developing the stain, 250 mL of chilled developer (1.5% NaOH, 1% sodium tetraborate and 1% formaldehyde) was added with agitation and then decanted when the first bands become visible. An additional 250 mL developer was added and agitation was continued until development was nearly completed. The developing reaction was stopped by adding fixing solution, and then the gel was rinsed thoroughly with d.i. water. Images were recorded by digital capture with a flatbed scanner.

Species-specific markers screening

Species-specific markers were screened using bulked segregate analysis (BSA), (Michelmore *et al.* 1996). The methods of BSA has three steps: 1) constructing each species primary gene pool (species gene pool): mixed DNAs were extracted from 50 seedlings which were from different provenances, and the specific markers for species would be screened with mixed DNAs; 2) constructing subgene pool (provenances gene pool): mixed DNAs were extracted from 10 seedlings in the same provenance. The reproductivity and conservation of specific markers screened from primary gene pool will be testified in this pool; 3) DNA sample (>30 samples) from individual seedling per provenance will be used to testify the specific markers again. If the marker is conservative within species and polymorphic intra-species, it is effective to identify species.

Results

Primers screening

The 23 of 276 (8.0%) microsatellite markers were amplified successfully in ten *pinus* species, and 5 of 23 (21.7%) were polymorphic (Table 1). Primer-pairs for microsatellite loci were obtained from *P. taeda* (3 loci of nuclear genome and 1 loci of chloroplast genome), *P. strobus* (1 loci of nuclear genome). Five pair-primers for identifying species were PtTX3013, PT45002, PtTX4092, PtTX4098 and RPS160 (Fig. 1). Some markers of 18 pair-primers were amplified, but none were polymorphic, or some were partially effective to ten species or polymorphic within-species. Therefore, they can not be used to identify species.

Table 1. Results of transfer of microsatellite loci

Marker	Primer sequences
PT45002	F) AAGTTGGATTTTACCCAGGTG
	R)GAACAAGAGGATTTTTTCTCATACA
PtTX3013	F) GCTTCTCCATTAACTAATTCTA)
	R) TCAAAATTGTTCGTAAAACCTC
RPS160	F)ACTAAGAACTCTCCCTCTCACC
	R)TCATTGTTCCCCAAATCAT
PtTX4092	F)GGTAGATACTTTCCATGAGTTAGG
	R)TCTAGTCCAGATCTTGGTCCAC
PtTX4098	F) GTGGGACCCCAAGCACT
	R) ATTGCCTCCTCTTTAGTCATCTCA

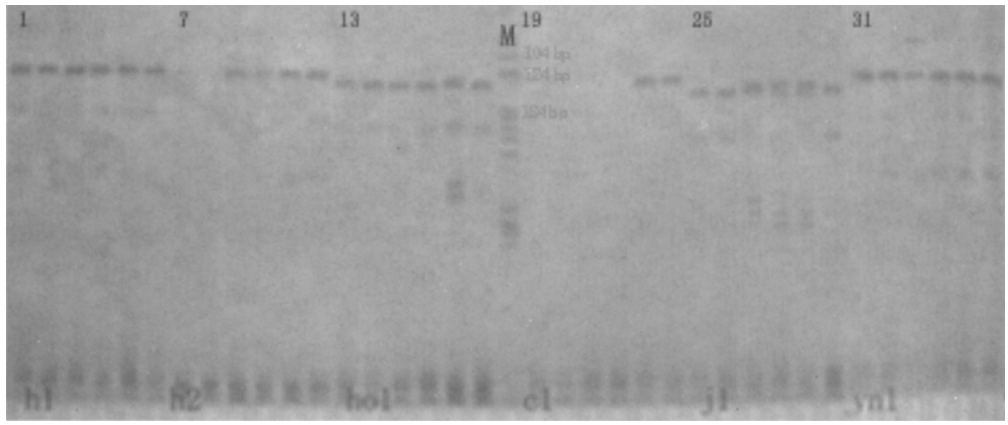

Fig. 1 Amplifying product by using PtTX3013

(Each lane represents one sample. 1-12 lanes belongs to *P. thunbergii* (h1 and h2 means seeds from different provenances) with a 124bp band; 13-18 belongs to *P. taeda* (ho1) with a 130bp band; M is Marker; 19-24 lane is *P. densiflora* (c1) with a 130bp band; 25-30 lane is *P. caribaea* (j1) with a specific band (160bp); 31-36 lane is *P. yunnanensis* (yn1) with a band 124bp)

Identifying seeds of *Pinus* species by using single primer and combination primers

The genome size of *Pinus* species is about 10×10^9-20×10^9 kilobases, in which there are 70%-80% highly repetitive sequences. Therefore, it is difficult to develop a specific marker to distinguish these species directly. However, SSR marker is specific for genus, especially for specific species. Though SSR primers developed from the specific species can transfer to other species in same genus, the portion of SSR primers that can produce band for all test species is still small. In this test, only 5 pair-primers were used to identify species and 2-4 polymorphic loci were amplified for each pair-primer. For some species, PCR product is specific with single pair-primer, for example, *P. tabulaeformis*

DNA amplified a specific locus (about 50 bp) primed with PtTX4098, *P. caribaea* amplified a size of 160 bp with the existence of primer PtTX3013 and *P. sylvestris* amplified a 140 bp fragment by using PT45002. For other species, information offered by combination primers (two or more primers) was enough to distinguish a species from others. For example, *P. taeda* should be distinguished from *P. caribaca* by using PtTX3013 firstly, then isolated from others by using PtTX4098 (Fig. 2).

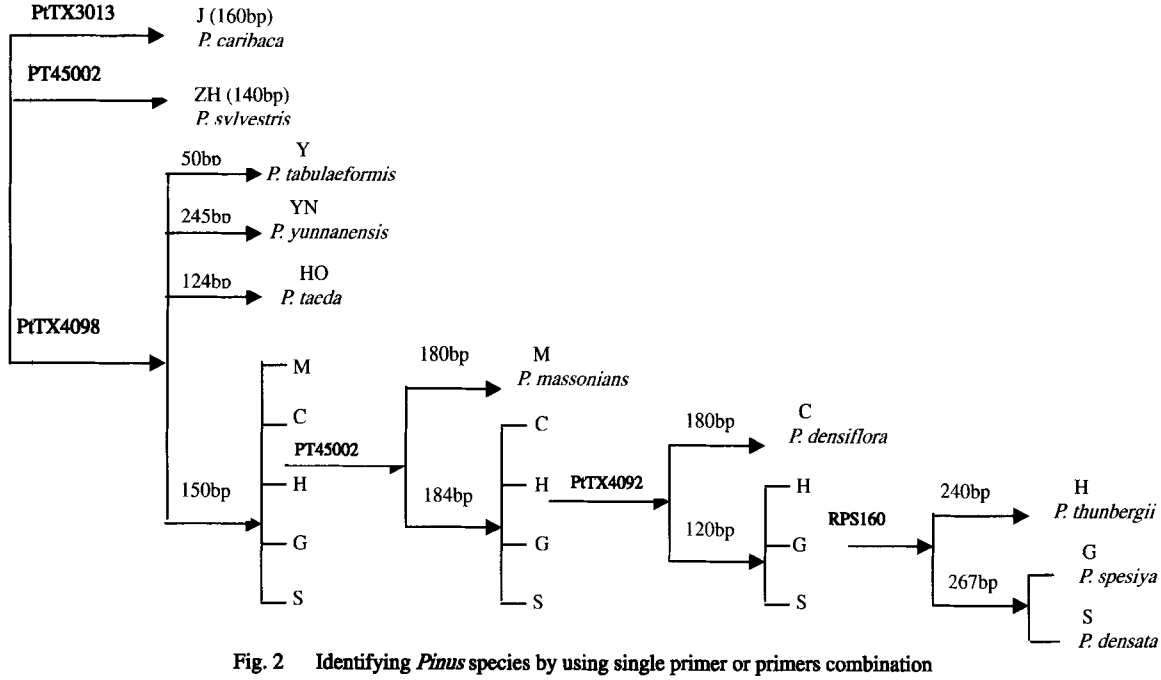

Fig. 2 Identifying *Pinus* species by using single primer or primers combination

The boldface words stands for SSR primer

Discussion

Transferring primers

Transferring SSR primers from related conifer species is attractive work. As is known, developing pine microsatellite primer has been proven to be difficult because the size and complexity (aprox. 75-86% highly repetitive DNA) of the pine genome represent significant barriers (Echt *et al.*1999; Smith and Devey 1994; Soranzo *et al.* 1998). Microsatellite transfer in pines has uncertain several reasons. Microsatellite transfer in flowering plants tends to be restricted to closely-related genera (Peakall *et al.* 1998). Similarly, transfer to other subgenera or non-*Pinus* coniferae has been reported to be poor (Echt *et al.* 1996; Fisher *et al.* 1998). Karhu *et al.* (2000) reported that optimization of polymerase chain reaction (PCR) conditions increased transfer rates for some of these loci.

In this study, though all primers screened were developed from *Pinaceae*, transfer rate was still low. The results screened included: i) Most of chloroplast SSR primers were transferred well without polymorphism across species. Only one chloroplast SSR (PT45002) was polymorphic in ten species. ii) Comparatively, transfer rate of nuclear SSR primers was lower than that of chloroplast SSR primers. Some of microsatellite marker can be transferred to all test species without polymorphism, or, some can transfer to part of test species. iii) SSR primers polymorphism was too high within species to identify *Pinus* species (e.g. PtTX3046). In fact, only 5 of 276 (1.8%) pair-primers screened were used to identify 10 *Pinus* species. The low transferring rate may be affected by two reasons. First, there was the far relationship between species SSR derived from and species SSR transferred to. The results of this paper indicated that three SSR markers developed from *P. taeda* (Sect. *Pinus*) were transferred to ten tested species belonging to Sect. *Pinus* well, and amplified highly polymorphic bands. However, no SSR markers derived from *Picea* or other genus was effective for *Pinus* species (Shepherd *et al.* 2002). Second, tri-nucleotide microsatellites from low-copy genomic regions increased transfer rates within *Pinaceae* (Kutil 2001). And transfer rates of microsatellite developed from genomic library, enriched low-copy library, cDNA library and other library were significantly different.

Among several molecular markers, SSR is the most effective marker to identify species because it can amplify 1-2 specific bands for every sample or species. The key difficulty is to develop specific SSR primer for specific species. Especially in Asia, the research work of SSR in pine and other coniferous tree is still undeveloped except of *Cryptomeria japonica* (Moriguchi, 2003). The research results indicated that 8 pine species were identified by using single primer, or two and more primers combination, however there were no effective SSR primers to identify *P. kesiya* and *P. densata*. SSR marker combining with other marker (such as RAPD that can be transferred SCAR marker) would be a better way to identify *Pinus* species completely.

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